

CHARACTERIZATION OF ENDOMETRITIS IN POSTPARTUM DAIRY COWS

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Abstract

Two experiments were designed to study endometritis in postpartum dairy cows. In the first experiment, 30 cows 28 to 41 days in milk (DIM) and without evidence of clinical endometritis were sampled using cytobrush cytology. Cytobrush sampling provided sufficient endometrial material to prepare cytologic specimens and to extract endometrial mRNA. Pro-inflammatory cytokines were analyzed in harvested endometrial tissue taken from cows with and without endometritis. Cytokine expression varied between experimental groups with 30-fold higher IL-6 expression levels ($P=0.01$), greater than 50-fold higher IL-8 expression levels ($P=0.0001$), and 20-fold higher TNF- α expression levels ($P=0.001$) in endometritis-positive versus negative cows. Regression analysis of cytokine expression levels (Ct) and the percentage of PMNs in subclinical endometritis-positive cows showed that for each additional threshold cycle required for IL-8 detection, which corresponded to two-fold less mRNA, the percentage of PMN decreased by 3.3% ($P=0.00001$). Similarly, for each additional threshold cycle required to detect IL-6 and TNF- α , the percentage of PMNs in endometritis-positive cows decreased by 2.3% ($P=0.015$) and 2.4% ($P=0.054$), respectively. Cows with $> 18\%$ PMNs required significantly fewer amplification cycles to detect IL-6 ($P = 0.01$), IL-8 ($P = 0.0001$) and TNF- α ($P=0.053$) mRNA than cows with $<18\%$ PMNs (endometritis-negative). There was a highly significant positive correlation between the expression of individual pro-inflammatory cytokines when comparing IL-8 and IL-6 ($P=0.0001$), IL-8 and TNF- α ($P=0.00001$), and finally IL-6 and TNF- α ($P=0.0002$).

In the second experiment, 340 cows 28 to 41 days in milk were examined using cytobrush cytology and transrectal ultrasonography of the uterus and ovaries. One-half of the cows were treated with benzathine cephapirin uterine infusion to determine the lowest PMN percentage where a significant improvement in reproductive performance occurred. Subclinical endometritis-positive ($>15\%$) cows in this study were defined as those with the lowest percentage of PMNs that was associated with a significant positive treatment effect. Treated cows with $>15\%$ PMNs required 31 fewer days ($P=0.041$) to become pregnant and had 2.5 times fewer services per conception ($P=0.0001$) than untreated cows with $>15\%$ PMNs. The likelihood of there being CLs at the time of examination in cows with $>15\%$ PMNs in endometrial cytobrush cytology was 2.3 times significantly higher ($P=0.04$). The treatment of cows with

ultrasonographically detectable fluid in the uterine lumen with benzathine cephapirin had no effect on days open compared to treatment of cows without fluid in the uterus ($P=0.39$). Cervical diameter and endometrial thicknesses did not differ between groups of cows with $>$, $<$ 15%PMNs ($P=0.46$, $P=0.36$, respectively).

In summary, based on the response to a single treatment with benzathine cephapirin, and the analysis of pro-inflammatory cytokine gene expression, we recommend that a threshold of $>18\%$ PMNs be used to define endometritis-positive disease status in cows 28 to 41 DIM. Cervical diameter, ultrasonographic evidence of uterine fluid and ultrasonographic measurement of endometrial thickness were not useful for diagnosing benzathine cephapirin responsive endometritis.

Key words: Endometritis, Cytokines, Treatment, Dairy cows

DEDICATION

To Oksana, Lina and my parents

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LIST OF ABBREVIATIONS

AI	Artificial Insemination
cDNA	Complementary DNA or Copy DNA
CL	Corpus luteum
cm	Centimeter
DIM	Days in Milk
FSH	Follicle-stimulating hormone
IL-1	Interleukin-1
IL-6	Interleukin-6
IL-8	Interleukin-8
IL-10	Interleukin-10
IU	Intrauterine
LH	Luteinizing hormone
LPS	Lipopolysaccharides
mg	Milligram
mL	Milliliter
mRNA	Messenger RNA
ng	Nano gram
ON	Ontario
PBS	Phosphate Buffer Saline
PGF2 α	Prostaglandin F2 α
PGFM	metabolite of PGF2 α
PMNs	Polymorphonuclear leukocytes
qRT-PCR	Quantitative real-time polymerase chain reaction
SD	Standard deviation
TLRs	Toll-like receptors
TNF- α	Tumor necrosis factor-alpha
μ g	Microgram
μ L	Micro liter
WCVM	Western College of Veterinary Medicine

1. GENERAL INTRODUCTION

According to Agriculture and Agri Food Canada, 30 to 40% of global agriculture entails livestock farming, which translates into financial returns of more than \$100 billion in Canada alone (www.agr.gc.ca). In 2010, there were 1.4 million cows on 20,624 dairy farms in Canada (www.agr.gc.ca). The typical Canadian dairy farm has 72 cows producing an average of 5,579 hectoliters of milk per year. Efficient dairy reproductive management based on current research findings is one of the many reasons for the success of the dairy industry.

A high level of reproductive efficiency requires each cow to be bred successfully, and calve with a calving interval that maximizes the output of milk within the herd (Groenendaal et al., 2004). Under normal circumstances, however, microbial contamination of the uterus is a frequent finding in postpartum dairy cows. The natural immune defense mechanisms within the uterus usually eliminate uterine infection by the time cows are inseminated, but in some cows, infection persists. When bacterial infection of the uterus persists beyond 4 weeks postpartum, the uterine infection is referred to as endometritis. Endometritis and subclinical endometritis are prevalent conditions in postpartum dairy cows resulting in substantial economic losses due to decreases in both milk production and fertility (Sheldon et al., 2004a). Therefore, the reproductive performance of a dairy cow after the voluntary waiting period is associated with her uterine health.

Interventions directed at improving reproductive management in dairy herds are most beneficial if they result in early identification of cows at increased risk of failure to become pregnant. A practical approach to identify cows at risk is to define the variables important in the case definition and to use reliable diagnostic technique(s) to identify cows with uterine infection/inflammation. Even though several screening tests have been developed and validated for their ability to identify cows with endometritis at an early stage, none of these techniques when used in isolation detect all of the affected cows (Barlund et al., 2008). Additional evidence-based studies are, therefore, required to

improve our ability to diagnose cows with endometritis and subclinical endometritis within dairy herds.

In addition to being successful in the identification of cows with uterine disease, an understanding of the underlying uterine immunology and its role in uterine involution and infection will contribute to the development of strategies to address disease and improve reproductive performance at the herd level. More research is necessary to advance our scientific knowledge about how bacterial infection affects the female reproductive tract to cause infertility or sub-fertility in cattle and the role of innate and acquired immunity in the disease process.

2. LITERATURE REVIEW

2.1-Postpartum Physiology

2.1.1-Uterine Involution

Uterine involution is a complex process that begins immediately after calving and involves uterine contractions, physical shrinking, necrosis, sloughing of caruncular material, and regeneration of the endometrium (Gier et al., 1968; Sheldon et al., 2004a). The number of days postpartum when the uterus is considered to be fully involuted varies among different studies. Morrow et al. (1966) reported that uterine involution is completed by 20 to 25 days postpartum, while another study indicated that only the epithelial regeneration is completed by that time whereas, the deeper layers of uterine tissue require 6 to 8 weeks to be fully restored (Sheldon et al., 2008). The cervix also undergoes shrinkage during uterine involution, but this process is slower than that of the uterus (Sheldon et al. 2006b).

Uterine lochia is a discharge that is evident immediately after calving and can be seen up to 12 to 18 days postpartum. It contains necrotized portions of uterine caruncles which slough off, fetal blood from the ruptured umbilicus as well as fetal fluids. Lochia is expelled from the postpartum uterus with help from myometrial contractions. Lochia is a normal physiologic discharge during uterine involution and must not be mistaken as indicative of pyometra or metritis (Dhaliwal et al., 2001; Sheldon et al., 2004b).

2.1.2- Contamination and Elimination of Bacteria from the Postpartum Uterus

In pregnant cows the vulva, vestibule, vagina and cervix function as anatomical barriers that protect the uterus from bacterial contamination during pregnancy. Relaxation of the vulva and cervical dilation during parturition allow for the entrance of bacteria into the uterus (Sheldon et al., 2004a; Azawi, 2008), therefore, bacterial contamination of the uterus postpartum is common. It has been demonstrated that 33% of dairy cows had positive bacterial cultures during the first week after calving and by the second week the

number of positive cases had increased to 44% (Fredriksson et al., 1985). More recent work, perhaps using more sensitive culturing techniques, has shown that the number of dairy cows with a uterine infection during the first 2 weeks postpartum is almost 80 to 100%, despite an uneventful calving (Sheldon et al., 2004a; Foldi et al., 2006).

Necrotized caruncles, blood and cell debris provide a perfect media for bacteria to grow during the immediate postpartum period. Under normal circumstances, the process of uterine involution effectively expels debris and encourages endometrial regeneration, so that the percentage of cows in which bacterial infection remains present at 3 weeks postpartum should decline to 40%; however, in approximately 10 to 17% of postpartum cows, conditions favouring bacterial growth persist and eventually cause endometritis (Sheldon et al., 2004a). Specific factors that may delay the elimination of bacteria from the postpartum uterus include the level and nature of the bacterial contamination, the degree of uterine involution, retention of fetal membranes, and the cow's immune status (Sheldon et al., 2006b).

2.1.3. Pathogenic Bacterial Infection of the Uterus

The bacterial agents commonly isolated from the uterus of postpartum cows are *Escherichia coli*, *Streptococci spp*, *Arcanobacterium pyogenes*, *Bacillus licheniformis*, *Prevotella spp* and *Fusobacterium necrophorum*. The most common pathogenic species are *Escherichia coli*, *Arcanobacterium pyogenes*, *Fusobacterium necrophorum* and *Prevotella* species (Foldi et al., 2006). Some bacteria, including *A. pyogenes*, *F. necrophorum* and *Prevotella spp.*, act synergistically to enhance the severity of uterine disease (Singh et al., 2008; Bonnett et al., 1991). Each of these species produces substances to enhance bacterial growth. *Fusobacterium necrophorum* actively invades uterine tissues and produces a leucocidal toxin that inhibits phagocytosis (Singh et al., 2008; Sheldon, 2004b). *A. pyogenes*, protected by the leucocidal toxin, in turn provides catalase and a growth factor which supports the proliferation of *F. necrophorum* (Singh et al., 2008). It has been reported that persistent infection with *A. pyogenes* after 21 days postpartum will reduce conception rates at the first postpartum service (Singh et al., 2008). Studies to evaluate the appearance and odor of vaginal mucus have shown that *A.*

pyogenes, *Proteus species* and *F. necrophorum* are associated with purulent or mucopurulent discharge evident in the vaginal mucus while *A. pyogenes*, *E. coli*, and non-hemolytic *Streptococci* are associated with foul smelling exudates (Williams et al., 2005).

2.1.4. Ovarian Activity in Postpartum Dairy Cows

During late gestation, the hypothalamic-pituitary axis is under the negative feedback effect of estrogens and progesterone produced by the placenta and ovaries. Therefore, the first ovulation occurs between 14 to 28 days postpartum (Yavas, 2000).

The return of ovarian cyclic activity postpartum both influences and depends on the uterine immune response. In fact, high concentrations of progesterone during the luteal phase suppress the immune response of the uterus and make the uterus more susceptible to bacterial infection (Lewis, 2003). Early ovulation and elevation of circulating progesterone concentrations before elimination of uterine bacterial contamination has been linked to the establishment of pyometra in postpartum cows (Olson et al., 1984; Sheldon et al., 2004b). Del Vecchio et al. (1994) showed that intrauterine infusions of *A. pyogenes* and *E.coli* in postpartum beef cows when progesterone concentrations were basal did not cause uterine infection whereas, all cows developed uterine infections when the bacteria were infused after the onset of luteal function and progesterone concentrations had begun to rise. However, the mechanisms underlying these observations are not clear. For instance, Subandrio et al. (2000) showed that blood or uterine neutrophil function in cattle is not consistently up-regulated by exogenous estradiol, nor suppressed by progesterone.

2.2. Immune Response of the Postpartum Uterus

The immune system plays a major role in uterine defense mechanisms; however, our understanding of this role is still greatly limited. Cellular defense against bacterial contaminants is provided by uterine leukocytes (Stossel, 1975), with PMNs, being the main leukocyte involved in bacterial clearance after uterine infection (Hussain 1989;

Gilbert et al., 2007). Polymorphonuclear cells recruited from the circulation to the infected uterus by chemotactic factors enhance phagocytosis of bacterial particles. It has been shown that the increase in leucocytes is followed by a reduction in total leucocyte counts one week postpartum, returning to higher levels over the following three weeks (Hussain et al., 1992; Cai et al., 1994; Mateus et al., 2002; Kim et al., 2005; Singh et al., 2008). A rise in cortisol levels during calving is the main cause of leukocytosis (Preisler et al., 2000); while the decline in leukocyte numbers has been associated with their migration into the uterine lumen and mammary glands (Singh et al., 2008).

It has been documented that bacterial contamination of the uterus in postpartum dairy cows is ubiquitous and the endometrium is the first line of defense against bacterial invaders (Galvão et al 2011; Sholden et al., 2004a). Receptors on endometrial cells and macrophages, called Toll-like receptors (TLRs), recognize highly conserved molecular patterns present on bacteria called pathogen-associated molecular patterns (PAMPs). Toll-like receptor binding of these PAMPs stimulates cells to produce and release pro-inflammatory cytokines and chemokines, including TNF- α , IL-6, and IL-8 (Tzianabos, 2000; Beutler et al., 2003). Interleukin-6 and TNF- α stimulate the production of anti-microbial peptides which assist in the elimination of pathogenic bacteria from the tissues. Fischer et al. (2010) showed that pro-inflammatory cytokines such as IL-6, IL-8, and TNF- α may accelerate PMN infiltration into the endometrium of cows following infection.

The role of TNF- α is to stimulate the expression of IL-8 and adhesion molecules on vascular endothelial cells. Interleukin-8 plays an important role in PMN and monocyte chemo-attraction and activation of PMNs and monocytes. These responses ultimately increase phagocytosis and bacterial killing (Roach et al., 2002).

Interleukin-6 is a pro-inflammatory cytokine that has different roles during the early stages of the inflammatory process. It activates mature PMNs and promotes PMN maturation, differentiation of monocytes into mature macrophages and differentiation of natural killer cells (Ishikawa et al., 2004). It is believed that increased expression of oxytocin receptors on myometrial cells can be induced by IL-6. Ishikawa et al. (2004) showed that IL-6 is present in the bovine uterus in high concentrations before parturition and decreases to baseline values by 8 days postpartum.

Zerbe et al. (2003) showed that infusing recombinant human IL-8 (rhIL-8) into the uterus of cows attracted PMNs into the uterus while anti-IL-8 monoclonal antibody treatment prevented PMN-dependent tissue damage as well as PMN infiltration during these conditions. These observations confirmed that IL-8 is an important chemo-attractant for PMNs. Although the presence of recombinant IL-8 in the uterus has been shown to increase the influx of PMNs and leucocytes into the bovine uterus, the question of whether IL-8 is generated in the uterus of infected cows is not clear.

Galvão et al. (2011) compared uterine endometrial cytokine gene expression between cows diagnosed with endometritis ($\geq 10\%$ PMNs in low-volume uterine lavage) at 35 days postpartum and cows without endometritis from calving to 7 weeks postpartum to evaluate differences in the pro-inflammatory cytokine production in cows without disease. Tumor necrosis factor- α gene expression during the first week postpartum was lower in cows with endometritis than control (healthy) cows. These investigators postulated that this lower TNF- α response may compromise the PMN and monocyte response to bacterial contamination of the uterus, decrease bacterial clearance, and contribute to the development of uterine disease. In contrast, IL-6 and IL-8 gene expression were elevated at 5 and 7 weeks after calving in cows with endometritis. Chapwanya et al. (2009) also reported that in uterine biopsies collected at 2 weeks postpartum, TNF- α expression was decreased in cows that were diagnosed with endometritis later in the postpartum period. However, other work examining endometrial cytobrush cytology samples collected between 3 and 4 weeks postpartum showed that both TNF- α , and IL-6 were usually increased in cows with endometritis (Gabler et al., 2009; Fischer et al., 2010). Therefore, the pro-inflammatory response to bacterial infection appears to change throughout the postpartum period.

Cow with endometritis may have a compromised ability to respond to bacterial contamination because pro-inflammatory cytokine gene expression is up-regulated later after calving than in their non-diseased counterparts. Decreased pro-inflammatory cytokine gene expression could lead to poor chemotaxis and activation of PMNs which would result in delayed bacterial clearance and development of endometritis (Galvão et al., 2011). In other words, healthy cows may have a greater pro-inflammatory response to bacteria invading the uterus after calving, and are therefore able to eliminate infection

successfully (Galvão et al., 2011). Nino-Soto et al. (2008) and Hammon et al. (2006) also postulated that lower expression of TNF- α and IL-6 in postpartum cows that developed endometritis could be due to an intrinsic defect in endometrial cell function or extrinsic mechanisms affecting endometrial cell activity such as a negative energy balance.

Beam et al. (1998) reported differences in cytokine gene expression between cows that developed endometritis and healthy cows when a negative energy balance was more pronounced. It was postulated that the ability of cows with endometritis to up-regulate expression of pro-inflammatory cytokine genes was affected by the condition of negative energy balance. Cows that developed endometritis due to severe negative energy balance may have an impaired ability to up-regulate pro-inflammatory cytokines in the first week after calving, but are able to mount an inflammatory response as soon as their nutritional status improves. The delayed uterine immune response and impaired leukocyte function that is related to a deficit in energy may also be complicated by increases in estradiol and cortisol that occur after calving (Goff et al., 1997; Wathes et al., 2009; Galvão et al., 2011).

Specific immunity by immunoglobulins derived locally from the uterus (IgA, IgG1, IgM) and from serum (IgG2) also contribute to uterine defense through enhanced bacterial opsonization and antigen presentation (Dhaliwal et al., 2001). Immunoglobulin G and IgM concentrations in lochia from healthy cows decrease after calving, but in cows with an abnormal puerperium, IgA and IgG concentrations in uterine fluids increase rapidly as endometritis develops (Aknazarov, 1998; Bondurant, 1999). These humoral immune mechanisms are also influenced by the steroid hormones reportedly increasing under the influence of elevated estrogen concentrations (Cobb et al., 1995). In the early postpartum period the low estrogen concentration environment which occurs prior to the resumption of cyclicity, predisposes the uterus to bacterial colonization by disarming the specific immune responses and leaving only non-specific responses such as neutrophil infiltration. However, the role of humoral immunity in bovine uterine disease is not fully understood (Lewis, 1997; Dhaliwal et al., 2001).

2.3. Definition of Uterine Diseases

There are different types or manifestations of uterine disease among postpartum dairy cows. Metritis, clinical endometritis, subclinical endometritis and pyometra are the types of uterine diseases most commonly reported.

Metritis has been defined as a uterine disease with systemic illness, fever and an enlarged uterus with a watery or purulent discharge. Metritis occurs most commonly during the puerperal period. There may be predisposing factors such as retained placentas, fetal maceration, or difficult calving (Foldi et al., 2006; Sheldon et al., 2006b; Chapwanya, 2008).

Clinical endometritis has been defined as a purulent or mucopurulent discharge or a cervical diameter >7.5 cm after 20 days in milk (DIM), or mucopurulent discharge after 26 DIM (LeBlanc et al., 2002b). Whether these cows have deeper uterine tissue involvement, higher degrees of bacterial contamination, a different or more extensive inflammation, or are cows that are experiencing a shortened inter-estrus interval which would facilitate an evacuation of uterine content has not been determined (Foldi et al., 2006).

Subclinical endometritis has been defined as the presence of inflammatory cells within the uterine lumen (PMNs), but without signs of clinical endometritis (Sheldon et al., 2006a; Foldi et al., 2006; Chapwanya, 2008). According to Sheldon et al. (2006a) these cows should also be >26 days postpartum so that the condition is not confused with normal uterine involution. The terms endometritis, usually referring to clinical endometritis, and subclinical endometritis are often used interchangeably. It can be speculated that the conditions are linked and are part of a continuum. Cows with clinical and subclinical endometritis do not show signs of systemic illness.

Pyometra is characterized by a collection of purulent exudate of variable amount within the uterine lumen. This condition is most likely to develop in cows that have their first postpartum ovulation before bacterial contamination of the uterus has been eliminated (Foldi et al., 2006; Chapwanya, 2008). Although there is functional closure of the cervix, the lumen is not always completely closed and some pus may discharge through the cervix into the vaginal lumen. Ultrasonographically pyometra is characterized by the presence of a CL on an ovary, an accumulation of fluid of mixed echodensity in the uterine lumen and distention of the uterus (Sheldon et al., 2006 b).

2.4. Diagnosis of Endometritis and Subclinical Endometritis

The diagnostic criteria for subclinical endometritis should identify cows at risk of pregnancy failure within an appropriate time. A variety of methods such as uterine palpation, ultrasonographic features of the uterus, vaginoscopy, endometrial cytology, uterine culture, and uterine biopsy has been reported to identify endometritis in postpartum cows (Barlund et al., 2008; Sheldon et al 2006b).

2.4.1. Uterine Palpation

Uterine palpation per-rectum has been the most frequently used method to diagnose endometritis (Sheldon et al., 2004a,b; Sheldon et al 2006b). Diagnosis of clinical endometritis by palpation per-rectum is a challenge because uterine size and palpable quality of content may vary between individuals and strongly depends on the stage of the postpartum period. It has been reported that diagnosis of clinical endometritis using palpation per-rectum is subjective, not effective and prone to error as it lacks standardization (Gilbert, 1992b; Foldi et al., 2006; Palmer, 2008).

2.4.2. Vaginoscopy

Vaginoscopy is an easy tool used to evaluate the vagina and cervix in postpartum cows and it should be employed as a routine diagnostic tool by veterinary practitioners especially if rectal palpation is the only other diagnostic modality being used (LeBlanc et al., 2002b). Barlund et al. (2008) reported that vaginoscopy lacked sensitivity when compared to endometrial cytobrush cytology for the diagnosis of both clinical and subclinical endometritis, but did support the use of vaginoscopy for the diagnosis of clinical endometritis in cows > 4 weeks postpartum. Because vaginoscopy is readily available to most veterinarians Barlund et al. (2008) encouraged the use of vaginoscopy as part of routine postpartum cow examination.

2.4.3. Ultrasonography

Ultrasonography has been used extensively as a diagnostic tool in veterinary medicine. Most of the research has been focused on the presence, volume and nature of uterine luminal fluid. Mateus et al. (2002) examined postpartum cows by per-rectum ultrasonography and concluded the volume of intrauterine fluid was significantly associated with impaired uterine involution and that the intrauterine fluid volume score was positively correlated with bacterial growth.

It seems reasonable that a local inflammatory response within the endometrium would result in some degree of tissue thickening. An attempt was made to use ultrasonographic measurement of endometrial thickness to diagnose endometritis in cows; however, it was shown that ultrasonographic measurement of endometrial thickness was not a very sensitive indicator of pregnancy status at 150 days (Barlund et al., 2008). Barlund et al. (2008) reported that endometrial thickness measurements >8 mm were less useful than endometrial cytobrush cytology with a sensitivity of 3.9% and a specificity of 89.2% compared to cytobrush cytology using a PMN threshold of >8% to diagnose subclinical endometritis between 28 and 41 days postpartum. Furthermore, Barlund et al. (2008) concluded that measurement of endometrial thickness could be easily influenced by the location on the uterine horn where the measurement was taken and the positioning of the ultrasound probe during the measurements.

2.4.4. Endometrial Cytology

Endometrial cytology has been used as a diagnostic tool in horses, but Kasimanickam et al. (2004) and Barlund et al. (2008) used a modified cytobrush to collect endometrial cytology samples in cows. The cytobrush is cut to 1 cm in length, threaded onto a solid stainless steel rod, 65 cm in length and 4 mm in diameter, and placed in a stainless steel tube, 50 cm in length and 5-6 mm in diameter, for passage through the cervix. The instrument is placed in a sanitary plastic sleeve to protect it from vaginal contamination. The vulva is cleaned with paper towels and the lubricated instrument is passed through the vagina to the external os of the cervix. The plastic sleeve is perforated and the stainless steel sheath and extension is manipulated through the

cervix and into the body of the uterus where the cytobrush is turned clockwise approximately $\frac{1}{4}$ turn to obtain endometrial cells from the uterus. The cytobrush is retracted into the stainless steel tube prior to removal from the uterus. The cytobrush is rolled onto a clean glass slide and allowed to air-dry on farm. Slides are stained with modified Wright Giemsa stain and then the slide is evaluated using 400x magnification and a differential count, using a minimum of 100 cells (endometrial, PMNs and squamous cells) is performed to provide a quantitative assessment of endometrial inflammation (Barlund et al., 2008).

The cytobrush technique has been used to evaluate the relationship between PMNs and conception. Various threshold proportions of PMNs (PMNs/ PMNs + endometrial cells) have been reported with greater proportions of PMNs being indicative of disease earlier in the postpartum period. In all studies, cows with a high percentage of PMNs were slower to conceive than those with low PMNs (McDougall et al., 2001a; Barlund et al., 2008; Gilbert et al., 2005). Dubuc et al. (2010) reported that $\geq 6\%$ PMNs or a mucopurulent vaginal discharge were the most the most appropriate indicators of endometritis in cows 35 ± 3 DIM whereas, $\geq 4\%$ PMNs was the most appropriate in cows 56 ± 3 DIM. Fischer et al. (2010) used a somewhat arbitrary $> 5\%$ PMN threshold to diagnose cows as having clinical and subclinical endometritis as recommended by Gilbert et al (2005). Barlund et al. (2008) used a $> 8\%$ threshold in cows with no evidence of vaginal discharge to define subclinical endometritis based on the relationship to 150-day pregnancy status. Kasimanickam et al. (2004) defined subclinical endometritis as the absence of visible purulent material in the vagina and a cytobrush cytology derived sample containing $> 18\%$ PMNs at 21-33 days postpartum, or $> 10\%$ PMNs at 34 and 47 days postpartum. Using 150-day pregnancy status as the outcome, Barlund et al. (2008) reported that the sensitivity of endometrial cytobrush cytology was only 12.9%, while the specificity was 89.9% in cows sampled 28 to 41 days postpartum. Such a low sensitivity and high specificity indicated that very few cows that were not pregnant at 150 days had $> 8\%$ PMNs, while most cows that were pregnant by 150 days had fewer than 8% PMNs indicating that were other factors unrelated to PMN proportion affecting pregnancy status at 150 days postpartum (Barlund et al., 2008). The different PMN ranges reported by the

various authors are indicative that days postpartum were considered as an important determinant of PMN levels.

The other methods for obtaining endometrial cytology include swab cytology, and low volume lavage. Swab samples were traditionally used for culture and/or cytology in herds with apparent infectious problems (Youngquist et al., 1997; Foldi et al., 2006; Sheldon et al., 2004a, b). Low volume uterine lavage (20 ml of sterile Sodium Chloride 0.9% or PBS) has been described as a tool for cytological examination of the uterus in dairy cows (Gilbert et al., 1998; Barlund et al., 2008). Gilbert et al. (2005) characterized subclinical endometritis as the incidence of >5% PMNs visible on a cytospin using low volume lavage at 40 to 60 days postpartum. Barlund et al. (2008) reported that lavage cytology and cytobrush cytology were essentially the same tests, however, the lavage results were less repeatable due to cellular distortion speculated to be caused by the infused saline or the centrifugation process.

2.4.5. Uterine Biopsy

Uterine biopsy and uterine bacteriological culture have been considered the gold standard diagnostic methods (Gilbert et al., 2005; Sheldon et al., 2006). However, uterine endometrial biopsy requires expensive equipment, and the clinical utility of that technique is limited due to the delay in acquiring results (Sheldon et al., 2004a,b; Etherington et al., 1988).

Some clinicians report the technique is time consuming, the procedure may negatively impact future fertility, and it is somewhat difficult to perform (Etherington et al., 1988; Gilbert et al., 2005). However, the technique has not been extensively evaluated to determine if indeed it is damaging to the uterus and in what time frame postpartum the negative effects occur, or if they always occur.

2.5. Treatment of Endometritis in the Dairy Cows

Sheldon et al. (1998) demonstrated that endometritis increased the calving to conception interval, decreased the pregnancy rate, and increased the culling rate. Therefore, the purpose of treating endometritis is to improve reproductive efficiency. That goal can be achieved by improving the uterine defenses and uterine clearance mechanisms, decreasing persistent infections and consequently reducing persistent inflammation in the treated uterus (McDougall, 2001b). Various therapeutic plans have been described with intrauterine antibiotic therapy being the most prevalent (Palmer, 2008). Limited research has been done to date to evaluate the use immunomodulators as a treatment for endometritis cows (Hussain et al., 1992; Subandrio et al., 1997; Singh et al., 2000).

The current treatment regimes for endometritis are based upon two different protocols: intrauterine infusion (IU) of antibiotics and administration of prostaglandin and its analogues. Other treatment regimes such as estrogen therapy are not as effective as prostaglandin injection or IU benzathine cephapirin therapy, and may impair future reproductive performance in cows (Palmer, 2008).

2.5. 1. Prostaglandin

Prostaglandin F2 α and its analogues have been used for the treatment of pyometra, as this hormone induces luteolysis in cows with luteal tissue, and also increases uterine contractility (Steffan et al., 1984; Bonnet et al., 1990; McDougall, 2001b). It has been documented that administration of prostaglandin in postpartum cows improved reproductive performance through three difference mechanisms, including increased uterine contractility, induced luteolysis, and enhanced phagocytic activity of uterine PMNs (Paisley et al., 1986; Galvão et al., 2009a). Kasimanickam et al. (2005a) showed a 70% improvement in the risk of becoming pregnant in cows with subclinical endometritis which were treated with cloprostenol between 20 and 33 days postpartum, compared to untreated controls. On the other hand, LeBlanc et al. (2002a) reported that prostaglandin administration in cows with clinical endometritis between 20 and 26 days postpartum and without a palpable CL, decreased the pregnancy rate whereas, using the

same product between 27 and 33 days postpartum cows resulted in an 18% improvement in pregnancy rate regardless of luteal status.

2.5.2. Intrauterine Therapy

The main purpose of intrauterine antibiotic infusion is to achieve a high level of antibiotic in the uterine lumen (Gilbert et al., 1992a). Agents used for intrauterine infusion include tetracycline (Sheldon et al., 1998), cephapirin (McDougall, 2001b), gentamicin (Daniels et al., 1976), and penicillin (Thurmond et al., 1993). However, it has been shown that using intrauterine antibiotic infusion therapy has some disadvantages such as interference with the phagocytic activity of PMNs, drug residues in milk, and development of bacterial resistance, and in many cases may be ineffective (Dinsmore et al., 1996; Gilbert et al., 1992a). The postpartum uterus is an anaerobic environment full of blood, tissue and other debris which make many antibiotics such as aminoglycosides ineffective (Paisley et al., 1986).

Oxytetracycline has been the most popular agent recommended for intrauterine infusion in cows; however, this agent is poorly absorbed into the deeper layers of the uterus (Bretzlaff et al., 1987). There are reports that drug concentrations were high enough in the caruncles and the endometrium 24 hours after intrauterine infusion of 5.5 mg/kg oxytetracycline, but concentrations were not sufficient in the myometrium and ovaries (Sheldon et al., 1998; Bretzlaff et al., 1987).

Use of a single administration of benzathine cephapirin (Metricure®, Intervet, Schering Plough, Canada, Ontario) improved the reproductive performance of cows with subclinical endometritis (diagnostic criteria: >18% PMNs using endometrial cytobrush cytology) (Kasimanickam et al., 2005a). Cephapirin is a first generation cephalosporin effective against most gram-positive and gram-negative bacteria in the uterus. Kasimanickam et al., (2005a) reported that cows with subclinical endometritis that received cephapirin between 20 and 33 DIM had an 89% increase in the risk of pregnancy compared to non-treated cows. Dohmen et al. (1995) reported that intrauterine infusion of cephapirin resulted in an 80% clinical cure rate and 60% bacterial clearance rate within 2 weeks of treatment. LeBlanc (2008) showed that using cephapirin in cows

27 and 33 days postpartum resulted in a 60% higher likelihood of those cows becoming pregnant and a 29% reduction in time to pregnancy compared to their untreated herd mates. McDougall (2003) randomly assigned 690 cows to receive cephalixin IU or no treatment at 41 ± 14 DIM. Cows treated with cephalixin were approximately 2 to 3 times more likely to be pregnant by 56 days into a seasonal breeding season.

Galvão et al. (2009b) demonstrated that intrauterine infusion with ceftiofur hydrochloride did not affect pregnancy rates by AI in cows treated at 44 ± 3 days postpartum, but did reduce the prevalence of uterine infection in cows, and the prevalence of *A. pyogenes*.

In conclusion, various studies have demonstrated that therapeutic strategies involving intrauterine antimicrobial treatment of uterine infections are effective when they involve the appropriate antibiotic at an adequate dose to maintain an effective endometrial concentration without any adverse effect on PMN function (Gustafsson et al., 1984; Thurmond et al., 1993; McDougall, 2001b).

2.6. The Use of Reproductive Performance Parameters in Dairy Cattle to Determine Endometritis Disease Status

Reproductive performance is one of the major determinants of the profitability of a dairy herd. Measures of reproductive performance and the statistical analysis of the data collected, should distinguish between management factors and physiological factors which impact the ability of a cow to become pregnant at an optimal time postpartum. Measures of reproductive performance have traditionally included such indirect parameters as time to first insemination, non-return rates, and calving interval (Stewart et al., 1994; LeBlanc et al., 2010). These methods, however, may be confounded by management factors such as the low intensity of estrus detection in herds, overestimation of the actual proportion of cows becoming pregnant, and inaccuracy in the measurement of reproductive performance, respectively (LeBlanc, 2010).

Assessment of the effect of one factor on reproductive performance may also be confounded by other factors including nutrition, parity of the cow, season of the year, body condition, herd management, heat detection, the use of reproductive management

programs, and disease incidence in the herd. Therefore, using a correct method for analysis of pregnancy data plays a crucial role in understanding the impact of certain factors on reproductive performance in dairy cattle. Survival analysis is the only valuable method for analysis of that data. Survival analysis measures the time to an event per individual subject and includes those subjects that do not experience the event of interest or are lost to follow-up during the study. Survival analysis also reduces the bias of losing information from cows that were culled or not pregnant at the end of study (Morton, 2006).

3. GENERAL HYPOTHESES

- 1- There is an association between endometrial pro-inflammatory cytokine gene expression (IL-6, IL-8, TNF- α) and endometrial cytology in postpartum cows.
- 2- A single intrauterine infusion with 500 mg benzathine cephapirin in postpartum dairy cows that are diagnosed as either clinically normal or with endometritis will result in improved reproductive performance and allow for the establishment of a new threshold proportion of PMNs in endometrial cytology samples for the diagnosis of endometritis based on the response to treatment.
- 3- Ultrasonography of the cervix and uterus in postpartum dairy cows is a useful tool for the diagnosis of endometritis.

4. Pro-inflammatory Cytokine Gene Expression in Endometrial Cytobrush Samples Harvested from Cows with and without Subclinical Endometritis

4.1. Abstract

A detailed understanding of the immune responses in the postpartum cow's uterus is lacking due to difficulties in obtaining endometrial cellular material. The objectives were to develop a minimally invasive cytobrush technique to collect endometrial cells in postpartum cows (28 to 41 days in milk, DIM) for the isolation of sufficient mRNA to characterize expression of key pro-inflammatory cytokines, including IL-6, IL-8 and TNF- α and their relationship to the percentage of polymorphonuclear leukocytes (PMN) in the corresponding cytosmears. Cows (n=30) without signs of clinical endometritis were categorized as inflammation-negative (n=18) or subclinical endometritis-positive (>18% PMNs; n=12) based on the endometrial cytobrush cytology. The cytobrush was rolled onto a slide for cytology before being transferred to a tube containing 1 ml Trizol® reagent and stored at -80°C until mRNA isolation. Each stained slide was examined three times (400x magnification) by an examiner blinded to sample identity and 100 cells per session were counted to determine the PMN percentage. Total RNA was extracted from each cytobrush sample and reverse transcribed to make cDNA. Quantitative real-time (qRT) PCR analysis of IL6, IL8, TNF- α and β -Actin gene expression was performed. Variables were percentage of PMNs in endometrial cytobrush cytology and relative mRNA expression levels for IL-6, IL-8 and TNF- α . Cytobrush sampling provided sufficient material to prepare cytosmears and extract high quality endometrial mRNA (mean=0.96 μ g RNA/sample). Cytokine expression varied between experimental groups with a 30-fold higher endometrial IL-6 expression levels ($P=0.01$) and a greater than 50-fold higher IL-8 expression level ($P=0.0001$) in subclinical endometritis-positive versus disease-negative cows. The TNF- α mRNA expression level was 20-fold higher ($P=0.001$) in subclinical endometritis-positive cows versus disease-negative cows. Regression analysis of IL-8 gene expression levels (Ct) versus PMN frequency showed that for each additional Ct required to detect IL-8 expression, the frequency of PMNs in the cytosmear decreased by 3.3% ($P=0.00001$). Similarly, for each additional Ct increase required to

detect IL-6 and TNF- α gene expression, the frequency of PMNs in cytosmears decreased by 2.3% ($P=0.015$) and 2.4% ($P=0.05$), respectively. There was a highly significant positive correlation between the expression of individual pro-inflammatory cytokines when comparing IL-8 and IL-6 ($P=0.0001$), IL-8 and TNF- α ($P=0.00001$), and finally IL-6 and TNF- α ($P=0.0002$).

In conclusion, the endometrial cytobrush technique can be used to obtain sufficient material for both cytology and RNA extraction, and the analysis of cytokine gene expression, especially IL-8, showed a strong correlation with endometrial inflammation in terms of percent PMNs.

4.2. Introduction

Postpartum endometritis caused by persistent bacterial infection has been shown to have a tremendous impact on the fertility of dairy cattle (Gilbert, et al., 2005; Williams, et al., 2005; Sheldon, et al., 2009; Barlund et al., 2008; Sheldon et al., 2006b). The most substantial effects of this disease are an increase in the number of days to conception, increased services per conception and an increased risk of culling (LeBlanc et al. 2002b; Gilbert et al., 1998). Because the effects of the disease are delayed, and often only detectable with deliberate statistical analysis, the economic significance of this disease remains largely unknown, but is speculated to exceed billions of dollars annually for the global dairy industry.

Polymorphonuclear cells (PMNs) are the first and most significant cell type recruited during uterine inflammation (Subandrio et al., 1997; Herath et al., 2006a; Rogan et al., 2005). Recent scientific publications have distinguished two types of endometritis: subclinical endometritis is defined as abnormally high numbers of PMNs in the uterine lumen; and clinical endometritis represents a PMN influx sufficient to result in purulent or mucopurulent exudates through the cervix (Gilbert et al., 2005, Sheldon et al., 2006b). Since only a portion of cows with endometritis display a visible discharge, and because PMN influx and visible discharge is associated with normal uterine involution, there has been a concerted effort to develop reliable diagnostic tools and protocols that could be used by both researchers and clinical veterinarians to diagnose subclinical endometritis

(Barlund et al., 2008, Kasimanickam et al., 2004, LeBlanc et al., 2002b, Gilbert et al., 2005, Sheldon et al., 2006b, Kasimanickam et al. 2005b, Dubuc et al. 2010). For example, Barlund et al. (2008) reported that endometrial cytobrush cytology was the most precise procedure for diagnosing subclinical endometritis in cows and that a threshold of >8% PMNs between 28 and 41 days postpartum was the lowest proportion of PMNs significantly affecting pregnancy status at 150 days postpartum. However, while the specificity in detecting subclinical endometritis was high at 89.9% the sensitivity was very low at 12.9% indicating that there were many other causes of non-pregnancy apart from cytologically detectable subclinical endometritis. Others have defined subclinical endometritis based on similar proportions of PMNs, but with a decreasing threshold as the postpartum period advanced (Kasimanickam et al., 2004, Dubuc et al., 2010). Despite these efforts the ability to predict future pregnancy status based on threshold proportions of PMNs has not improved (Dubuc et al., 2010) which reinforces the need for a better understanding of postpartum uterine inflammation.

Following infection, numerous pro-inflammatory cytokines and chemokines, including tumor necrosis factor- α (TNF- α) and interleukins (IL) -6, and -8, are produced in the uterus (Herath et al. 2006b, Fischer et al. 2010). Interleukin-8 is a potent chemotactic factor recruiting PMNs to the site of inflammation (Zerbe et al., 2003, Hoch et al., 1996, Mette et al., 2010) and both IL-8 and TNF- α concentrations were elevated in uterine tissue within a few hours of bacterial infusion into the equine uterus (Mette et al., 2010). Elevated interleukin-6 expression has been detected in uterine biopsies from both mares (Fumoso et al., 2003, Fumoso et al., 2007) and cows (Ishikawa et al., 2004) susceptible to developing subclinical endometritis. Gabler et al. (2009) were the first to report that endometrial cells collected with a cytobrush could be used to analyze pro-inflammatory gene expression. This approach eliminated the need for more invasive endometrial biopsies. A subsequent publication (Fischer et al., 2010) reported increased IL-8 and TNF- α expression in the bovine uterus associated with clinical and subclinical endometritis suggesting that a possible diagnostic threshold level may exist. Therefore, it seems plausible that the assessment of one or more pro-inflammatory cytokines may provide more reliable diagnostic indicators of acute subclinical endometritis than PMN percentages.

The objectives of this study were: 1) To further evaluate the cytobrush technique as a non-invasive method to collect sufficient endometrial cells for both cytology and mRNA isolation from postpartum cows with subclinical endometritis; 2) To evaluate the association between pro-inflammatory cytokine gene expression (IL-6, IL-8, TNF- α) and endometrial PMN percentages.

4.3. Materials and Methods

4.3.1. Animals

The study population consisted of 30 Holstein cows (11 primiparous; 19 multiparous) from six commercial dairy herds receiving veterinary service from the Western College of Veterinary Medicine (WCVN). All cows appeared healthy at the time of examination and had body condition scores > 2.5 (scale 1 to 5). Cows between 28 and 41 days postpartum were identified prior to regularly scheduled herd visits using herd records maintained by the WCVN. None of the cows included in the study showed evidence of clinical endometritis defined as purulent or mucopurulent uterine discharge (Sheldon et al., 2006b).

This project was approved by the University of Saskatchewan, University Committee on Animal Care and Supply.

4.3.2. Sample Collection and Endometrial Cytobrush Cytology

A Cytobrush Plus GT (Medscand Medicinal, Sweden) instrument was modified to enable the collection of endometrial cellular material. Briefly, the modification of the Cytobrush plus GT consisted of shortening the handle to 1 cm and threading it so it could be screwed into a stainless steel rod (65 cm in length, 4 mm outside diameter) as described by Barlund et al. (2008). All modifications to the cytobrush were performed while the brush portion was retained within its original packaging (shipped in packages of 10) to prevent cytobrush contamination with cellular material from other sources.

Prior to each farm visit an appropriate number of cytobrush units were assembled based on the number of cows to be sampled. Firstly, a stainless steel rod and a stainless steel sheath (50 cm long, 6 mm outside diameter) designed to cover the cytobrush and rod during passage through the vagina and cervix were removed from chemical sterilization media (CidexPlus®, Johnson & Johnson, Markham, Ontario) and rinsed with 20 mL sterile saline (Sodium Chloride 0.9%, Abbott Laboratories Ltd., Saint Laurent, Quebec). The rod was then placed inside the guard and using sterilized thumb forceps a modified cytobrush was removed from its packaging and threaded into the rod. A plastic sheath (Chemise Sanitaires, IMV Technologies, France) was then placed over the assembled unit which was then placed inside a clean and sealable container for transport to the farm.

Cattle to be sampled were suitably restrained. A sterile lubricant (Triad Sterile Lubricating Jelly, H&P Industries Inc., Mukwonago, WI, USA) was applied to the tip of the plastic sheath, the labia were gently parted and the double guarded cytobrush was inserted into the vagina to a point approximately half of the way between the vulva and the cervix. A sleeved arm was then introduced into the rectum to empty it of feces and to facilitate manipulation of the instrument into the anterior vagina and through the cervix. At the external os of the cervix, the outer plastic sleeve was perforated and the stainless steel sheath and extension were passed through the cervix.

Upon reaching the innermost ring of the cervix the cytobrush was advanced approximately 1 cm beyond the sheath into the lumen of the uterine body where it was rotated clockwise a full 360 degrees to obtain cellular material from the endometrium. The cytobrush and rod were retracted inside the guard and carefully removed from the reproductive tract. While still cow-side, the cytobrush was gently rolled onto a clean microscope slide utilizing only half of its entire circumference so as to ensure that a suitable quantity of cellular material remained on the untouched surface for cytokine analysis. Slides were air-dried and packaged for transport to the lab. Next, using thumb forceps, the cytobrush was carefully detached from the rod mechanism and placed in a sterile tube containing 1 ml Trizol® reagent (Invitrogen, Burlington, ON).

Immediately following the on-farm processing of cytobrush material, each cow underwent a trans-rectal ultrasound examination (Sonosite VET 180 Plus, Bothell, WA,

USA) of the uterus and ovaries to determine the presence or absence of uterine fluid and ovarian structures including corpora lutea (CLs), respectively.

Cytology smears were stained with modified Wright Giemsa Stain using an automated slide stainer (Hema-tek slide Stainer; Miles Scientific, Napierville, IL, USA). All slides were examined using light microscopy at 400x magnification to identify individual cell types, including endometrial epithelial cells, and PMNs. Polymorphonuclear cell counts were expressed as the proportion PMNs counted out of combined PMNs plus endometrial cells. A total of 300 cells were counted by a single examiner. Cows were categorized as subclinical endometritis-positive or subclinical endometritis-negative on the basis of the percent PMNs/cytosmear.

4.3.3. RNA Isolation and Reverse Transcription

Total RNA was extracted from uterine tissue samples preserved in Trizol® reagent (Invitrogen) according to the manufacturer's instructions. Briefly, samples were thawed, placed on ice, and 0.2 mL of chloroform was added per 1 mL of Trizol® reagent. Sample tubes were securely capped, briefly vortexed, placed on ice for 2 min, and then centrifuged at 12,000 x g for 15 min at 4 °C. The aqueous phase was then transferred to a fresh tube and RNA was precipitated by mixing with 550 µL isopropyl alcohol and 2.2 µL of glycogen (Roche). Samples were incubated -70 °C for 10 min and centrifugation was repeated before removing the supernatant. The RNA was first washed with 1 mL 100% ethanol and then 70% ethanol. The final RNA pellet was dried for 15 min at room temperature and then dissolved in 20 µL of RNAase-free water by repetitive gentle pipetting. RNA integrity was verified using the Agilent 2100 Bioanalyzer (G2938B, Agilent technologies, Inc).

4.3.4. Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Quantitative RT-PCR (qRT-PCR) was used to assess cytokine expression in the cytobrush samples. First, cDNA was synthesized using the qScript™ cDNA Synthesis Kit (Qiagen, Mississauga, ON) according to the manufacturer's instructions. Briefly, 500

ng of total RNA was reverse transcribed by adding 4 μ L of qScript reaction mix, 1.0 μ L of qScript RT and nuclease free water to a final volume of 20 μ L. The cDNA was prepared in the GeneAmp PCR System 9700 (Applied Biosystems) using the following program: 1 cycle at 22 °C/5 min, 1 cycle at 42 °C/30 min and 1 cycle at 85 °C/5 min, at the end of the run samples were stored at -20 °C. The PerfeCta™ SYBR® Green for iQ™ Kit (Invitrogen) was used according to the manufacturer's instructions to perform qRT-PCR analysis of IL6, IL8, TNF- α and β -actin transcript frequency expression. Briefly, 9 μ L of PerfeCta™ SYBR® Green master mix for iQ (2X), 3 μ L of the appropriate primer set (Table 4.1; Wilson et al. 2007) at 3.3 μ M, plus 3 μ L of cDNA (~10 ng) template were added for a final reaction volume of 15 μ L.

Gene	Accession Number	Primer Direction	Primer Sequence (5'-3')
IL-6	BC123577	Forward	CCAGGAACGAAAGAGAGC
		Reverse	CAGAAGTCATCACCAGGAG
IL-8	EU276073	Forward	CAAGAGCCAGAAGAAACCTGAC
		Reverse	AGTGTGGCCCACTCTCAATAAC
TNF-α	EU276079	Forward	CTCTTCTGCCTGCTGCACTTC
		Reverse	CCATGAGGGCATTGGCATAACG
β-actin	AY141970	Forward	AGGCATCCTGACCCCTCAAGTA
		Reverse	GCTCGTTGTAGAAGGTGTGGT

Table 4.1: Primer sequences for qRT-PCR amplification of mRNA

The reaction was performed in the iCycler iQ RT-PCR detection system (Bio-Rad) using the following program: 1 cycle at 50 °C/2 min, 1 cycle at 95 °C/30 sec, 45 cycles of: 95 °C/15 sec, 55 °C/30 sec and 72 °C/30 sec. After cycling, the temperature was increased starting from 56 °C at a rate of 1 °C every 10 sec to generate a melting curve (40 times). The amplification data obtained by the qRT-PCR for individual genes was expressed as Cycle threshold (Ct) and was subtracted from the Ct for β -actin to obtain the Δ Ct where [Δ Ct = Ct (Cytokine) – Ct (β -actin)].

4.3.5. Statistical Analyses

All statistical analyses were performed using STATA-10 (Stata, Version 10-0, StataCorp LP, Texas) and differences were considered significant when the *P*-value was <0.05.

The Shapiro-Wilk test was used to determine if the data in both groups were normally distributed ($P > 0.05$). Gene expression data was stratified into two different groups based on changing the threshold percentage of PMNs in cytosmeareds (>8%, >11%, >15%, and >18%) to define subclinical endometritis-positive and subclinical endometritis-negative groups. The > 8, >11 and >18 % thresholds were selected based on previous published experiments Barlund et al., 2008; Dourey et al., 2011; Kasimanickam et al., 2004, respectively where they were reported to be indicative of subclinical endometritis-positive disease status. The >15% threshold was selected basis on the findings of a related project (Ghasemi et al., unpublished) contained in this thesis. This data stratification was used to determine how well relative expression levels (Ct) for individual cytokines (IL-6, IL-8, and TNF- α) correlated with the definition of subclinical endometritis.

Spearman's rank correlation coefficient was used to describe the relationship between the proportion of PMNs on the endometrial cytosmear and the level of IL-6, IL-8, and TNF- α expression in qRT-PCR. Linear regression analyses of the correlation between the expression of endometrial pro-inflammatory cytokines (IL-8 and IL-6), (IL-8 and TNF- α), and (IL-6 and TNF- α) were performed to determine if there was a significant correlation within cytokine groupings.

The Wilcoxon signed-rank test was used to evaluate whether the presence of a CL at the time of sampling had a significant effect on the quantity of RNA isolated.

4.4. Results

Twelve cows were classified as subclinical endometritis-positive and 18 cows were classified as subclinical endometritis-negative based on endometrial cytobrush

cytology when using a threshold of >18% PMNs. None of the 30 cows had ultrasonographically detectable uterine fluid. A threshold of >18% PMNs in endometrial cytobrush cytology 28 to 41 days after parturition was shown to be the lowest PMN percentage which was significantly associated with an elevation of all three endometrial pro-inflammatory cytokines, IL-6, IL-8 and TNF- α .

The mean (\pm 1 SD) quantity of total RNA isolated from endometrial samples was 0.97 ± 1.05 $\mu\text{g}/\mu\text{L}$ and 0.94 ± 0.14 $\mu\text{g}/\mu\text{L}$ for cows diagnosed with and without subclinical endometritis, respectively, and was sufficient for gene expression analysis. The quantity of RNA isolated was not significantly different between groups or among cows within each group ($P=0.93$).

Twenty of the cows had ultrasonographically detectable luteal tissue whereas, the remaining 8 cows had evidence of follicular activity, but no CLs. Total RNA recovery was not influenced by the presence or absence of a CL ($P=0.12$).

4.4.1. Pro-inflammatory Cytokine mRNA Expression

The levels of pro-inflammatory cytokine gene expression for individual cows are presented in Figure 4.1. Data are reported as the change in threshold cycle (ΔCt) relative to the house-keeping gene, β -actin. There was no significant difference in β -actin gene expression between groups ($P=0.3$), with average Ct values of 23.3 and 24.1 in subclinical endometritis-positive and negative cows, respectively ($P=0.12$). The consistency of β -actin expression confirms its suitability as a reference gene.

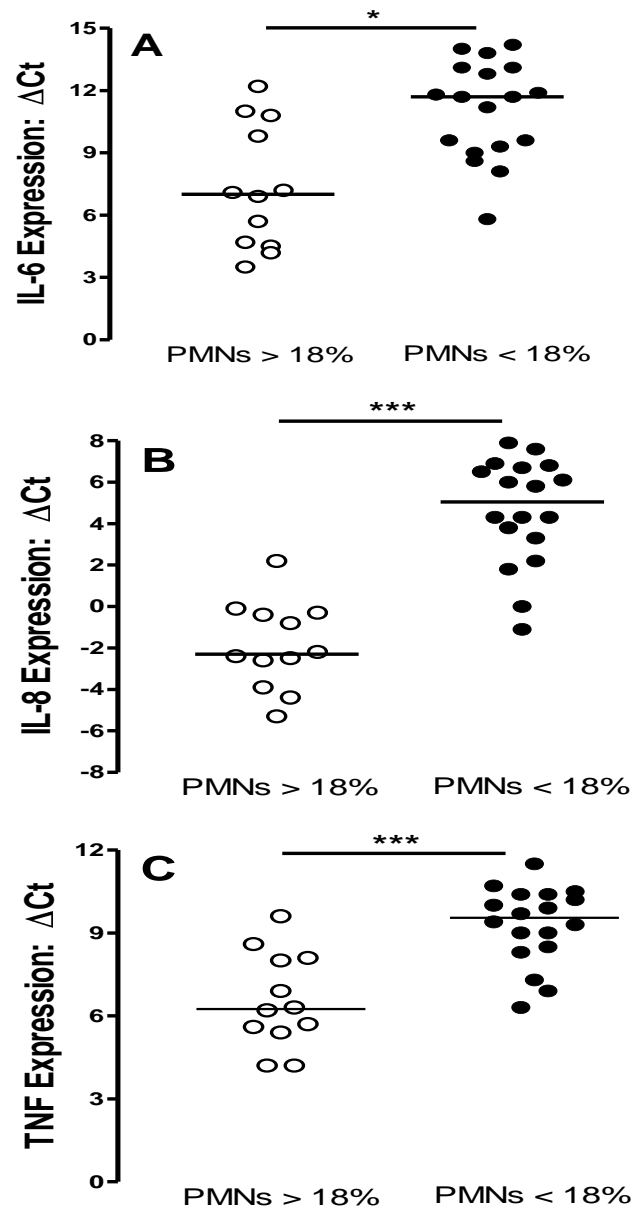


Figure 4.1: Cytokine gene expression levels shown as a change in cycle threshold relative to β -actin (ΔCt). Values are presented for IL-6 (**A**), IL-8 (**B**), and TNF- α (**C**). Uterine endometrial tissue was obtained using a cytobrush technique to collect samples from subclinical endometritis-positive (n=12) and subclinical endometritis-negative (n=18) cows. Subclinical endometritis-positive cows were identified by endometrial cytospins with >18% PMNs. Data presented are values for individual animals and the

horizontal line represents the median value for each group.*Significant differences between groups ($P<0.05$). ***Significant differences between groups ($P<0.01$).

Interleukin-6, IL-8, and TNF- α mRNA was detected in all samples and cows with >18% PMNs required significantly fewer amplification cycles (lower ΔC_t values) to detect IL-6 ($P=0.01$), IL-8 ($P=0.0001$) and TNF- α ($P=0.053$) mRNA than subclinical endometritis-negative cows. Each threshold cycle represented a 2-fold change in gene expression and a decrease in one ΔC_t represented a doubling of gene expression.

The change in cytokine gene expression with increasing numbers of PMNs varied considerably among individual cytokines (Table 4.2).

Variable % PMNs	Selected cytokines	Risk ratio	<i>P</i> Value
>8 (n=22)	IL-6	0.001	0.89
	IL-8	0.0005	0.94
	TNF- α	0.086	0.38
>11 (n=16)	IL-6	0.031	0.57
	IL-8	0.0001	0.99
	TNF- α	0.011	0.73
>15 (n=14)	IL-6	0.23	0.08
	IL-8	0.27	0.056
	TNF- α	0.12	0.22
>18 (n=12)	IL-6	0.19	0.01
	IL-8	0.52	0.0001
	TNF- α	0.12	0.053

Table 4.2: Regression analysis of endometrial cytokine gene expression and PMN thresholds in cytobrush cytologies.

For example, endometrial IL-6 and IL-8 displayed 30-fold ($P=0.01$) and 50-fold ($P=0.0001$) higher median expression levels, respectively, in subclinical endometritis-positive compared to subclinical endometritis-negative cows. The difference in TNF- α gene expression was less substantial with subclinical endometritis-positive cows showing a 20-fold ($P=0.001$) higher median expression level than subclinical endometritis-negative cows.

Regression analysis of IL-8 gene expression levels (Ct) versus PMN frequency showed that for each additional Ct required to detect IL-8 expression, the frequency of endometrial PMNs in the cytosmear decreased by 3.3% ($P=0.00001$). Similarly, for each additional Ct increase required to detect IL-6 and TNF- α gene expression, the frequency of PMNs in the cytosmeas decreased by 2.3% ($P=0.015$) and 2.4% ($P=0.05$), respectively (Fig 4.2).

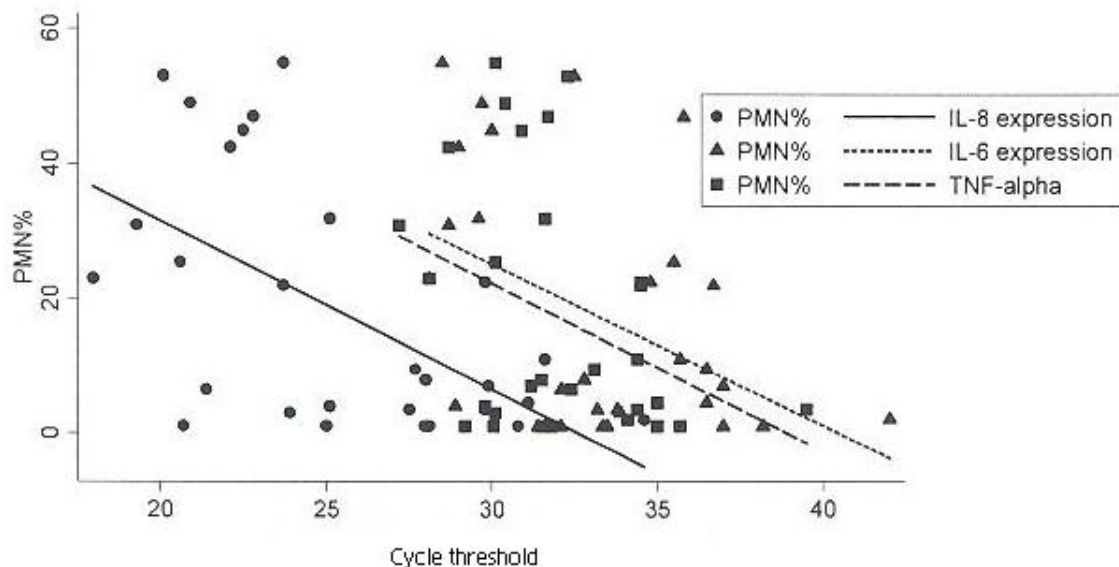


Figure 4.2. Linear regression analysis of the correlation between the percentages of PMNs in endometrial cytosmeas versus Ct required to detect IL-8, IL-6, and TNF- α gene expression in endometrial tissue.

In addition, there was a very positive correlation between the expression of individual endometrial pro-inflammatory cytokines when comparing IL-8 and IL-6 ($P=0.0001$), IL-8 and TNF- α ($P=0.00001$), and finally IL-6 and TNF- α ($P=0.0002$) (Table 4.3).

	IL-8	IL-6	TNF-α
IL-8	-	0.448	0.464
IL-6	0.448	-	0.397
TNF-α	0.464	0.397	-

Table 4.3: Correlation coefficient (R^2) between mRNA expression of selected pro-inflammatory cytokines in endometrial samples collected using a cytobrush from cows between 28 and 41 days postpartum ($P \leq 0.0002$).

In particular, IL-8 mRNA expression was highly correlated with the expression of IL-6, and TNF- α . The mRNA expression of IL-6 with TNF- α was also highly correlated (Fig 4.3 A, B).

Figure 4.3A

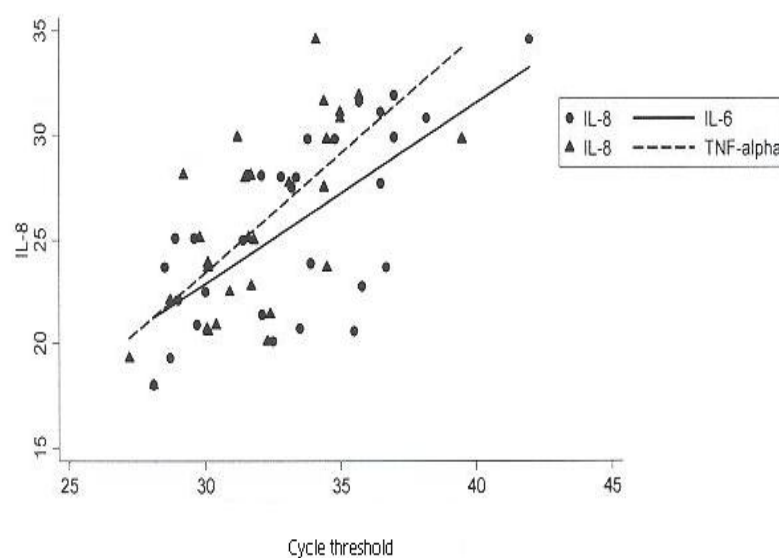


Figure 4.3B

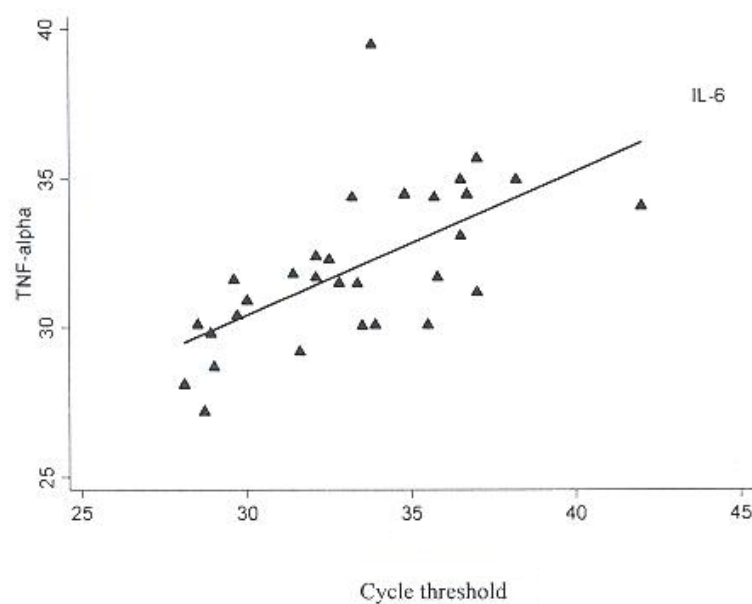


Figure 4.3 (A, B): Linear regression analyses of the correlation between the expressions of individual pro-inflammatory cytokines. There was a significant correlation between IL-8 and IL-6 ($P=0.0001$), (IL-8 and TNF- α) ($P=0.00001$) (A), and (IL-6 and TNF- α) ($P=0.0002$) (B).

4.5. Discussion

The cytobrush technique has been shown to be useful to detect subclinical endometritis in cows (Kasimanickam et al., 2004; Barlund et al., 2008), and more recently has been described as a method to collect samples to isolate RNA of sufficient quantity and quality for gene expression analysis (Gabler et al., 2009; Fischer et al., 2010). Previously, endometrial tissue collection for gene expression analysis had been performed only by endometrial biopsy, which was reported to have adverse effects on reproductive performance in cattle (Etherington et al., 1988). An additional concern with endometrial biopsy is the potential for the tissue samples to include multiple layers of the uterus which may influence the interpretation and sensitivity of gene expression data. In the current study, cytological examination of cytobrush samples confirmed specific recovery of primarily endometrial epithelial cells and infiltrating PMNs. The cytobrush technique provides a much less invasive method to harvest endometrial epithelial cells and provided sufficient high quality RNA to analyze gene expression at the interface between the host and the uterine environment. Therefore, the cytobrush technique can be considered a safe and effective method to harvest sufficient cellular material from the bovine endometrium for both diagnostic and experimental purposes. Furthermore, the amount of RNA isolated from cytobrush samples was not influenced by the presence or absence of an ultrasonographically-detectable CL which strongly suggests that IL-6, IL-8, and TNF- α gene expression is not influenced by the stage of the estrous cycle.

In the current study, various PMN percentages (>8%, >11%, >15%, and >18%) in cytobrush samples were examined for their relationship to the level of pro-inflammatory cytokine mRNA expression at 4 weeks (31 ± 1) post-calving. A threshold of >18% PMNs in endometrial cytobrush cytology 28 to 41 days after parturition was the lowest percentage of PMNs significantly associated with an elevation of all three pro-inflammatory cytokines, IL-6, IL-8 and TNF- α . The very positive correlation between IL-8 expression levels and the threshold of >18% PMNs strongly supports the conclusion that this endometrial cytobrush cytology threshold is an appropriate indicator of uterine inflammation for the diagnosis of subclinical endometritis in cows at this stage in the postpartum interval.

Findings in the current study support the concept that pro-inflammatory cytokines play a role in PMN recruitment and infiltration of the endometrium following infection as previously reported by Fischer et al. (2010). The highest correlation observed was between IL-8 and PMN frequency in the uterine samples of cows with subclinical endometritis (Figure 4.2). The 50-fold increase in IL-8 observed in cows with subclinical endometritis (Figure 4.1B) may also be biologically important since IL-8 is a potent chemokine regulating PMN recruitment (Zerbe et al., 2003). Robertson (2005) reported that seminal plasma was able to stimulate IL-8 expression in endometrial epithelial cells in women and mice suggesting that IL-8 may modulate the timely recruitment of PMNs to the endometrium. Furthermore, Zerbe et al. (2003) showed that an infusion of human recombinant interleukin-8 (rhIL-8) into the uterus of mares and cows resulted in PMN recruitment, while anti-IL-8 treatment prevented PMN-dependent tissue damage and PMN infiltration. Together, these results suggest that IL-8 plays a causative role in acute inflammation by recruiting and activating PMNs. Indeed, a greater inflammatory response to bacterial infections occurs in cows that develop persistent (chronic) subclinical endometritis and become sub-fertile (Walsh et al., 2011). In the current study, significantly greater IL-8 expression levels were consistently observed at 4 weeks postpartum in cows diagnosed with subclinical endometritis when compared with IL-8 expression levels from cows with a low percentage of PMNs. These results are similar to findings reported by Fischer et al. (2010) for cows sampled 21-27 days postpartum. Galvão et al. (2011) also reported significantly increased IL-8 gene expression in cows diagnosed with subclinical endometritis ($\geq 10\%$ PMNs at 5 weeks and $\geq 8\%$ PMNs at 7 weeks) using low-volume uterine lavage with a peak level of IL-8 expression at 7 weeks after calving. Data generated in the present study confirm that in cows with subclinical endometritis, IL-8 gene expression by 4 weeks postpartum is higher than the other pro-inflammatory cytokines examined. Therefore, once inflammation is established in the uterus, the expression levels of IL-8 may most accurately reflect the infection status of the cow.

Interleukin-6 gene expression was elevated 30-fold at four weeks postpartum in cows with $>18\%$ PMNs when compared to cows with $<18\%$ PMNs (Figure 4.1A); albeit less than the 50-fold increase of IL-8. Interleukin-6 is a pro-inflammatory cytokine

produced during the early stages of inflammation with a broad range of activities, including PMN maturation and activation, differentiation of monocytes into mature macrophages, and differentiation of natural killer cells (Ishikawa et al., 2004). Ishikawa et al. (2004) reported that IL-6 expression was elevated in the bovine uterus before parturition and decreased to baseline values by 8 days after parturition. Interleukin-6 and IL-8 gene expression were highly correlated in the individual cows in the present study, which suggests close integration of pro-inflammatory cytokine expression when the inflammatory cascade is activated in response to bacterial infection. Galvão et al. (2011) also reported increased IL-6 gene expression during the first and seventh weeks postpartum in cows with subclinical endometritis. However, using a >5% PMN threshold in endometrial cytobrush cytology to diagnose subclinical endometritis, Fischer et al. (2010) reported IL-6 expression was not influenced by inflammation between 21 and 27 days postpartum. Discrepancies between the results of the current study and previous studies of IL-6 expression at 4 weeks postpartum may be explained by differences in either sampling technique (biopsy versus cytobrush) and the percentage of PMNs (>5%, $\geq 8\%$, $\geq 10\%$ versus >18%) used to define a subclinical endometritis-positive disease status. Epithelial cells are the primary sensors of invading pathogens at mucosal surfaces (Herath et al., 2009) and endometrial cytobrush cytology directly targets the endometrial epithelium which may increase the level of mRNA isolated. .

Tumor necrosis factor- α expression was 20-fold greater at 4 weeks postpartum in cows with >18% PMNs when compared to cows with <18% PMNs (Figure 4.1C). Fischer et al., (2010) reported a statistically significant 2-fold increase in TNF- α expression at 21-27 days postpartum when comparing cows with subclinical endometritis versus healthy cows. The 10-fold difference between the results of the current study and those of Fischer et al. (2010) may be explained by the use of a different PMN threshold to define subclinical endometritis, and the stage of the postpartum period when the cows were sampled. Tumor necrosis factor- α is produced by various types of immune cells, but especially PMNs during inflammatory processes (Carswell et al., 1975; Hunt et al., 1992; Worku et al., 2009). As well, TNF- α is produced by epithelial, glandular epithelial, and endothelial cells in the stromal layer of the bovine uterus (Okuda et al. 2010) and is involved not only in regulating

immunologic, inflammatory or reparative responses, but also controls prostaglandin (PG) synthesis within the bovine endometrium (Okuda et al., 2004). Galvão et al. (2011) evaluated the modulation of TNF- α gene expression during the postpartum period in dairy cows and reported that expression was altered in cows diagnosed with subclinical endometritis at 5 weeks after calving. Cows with subclinical endometritis had lower levels of TNF- α expression when compared to healthy cows 1 week postpartum, but TNF- α expression increased significantly after 5 weeks postpartum. This observation is not consistent with the findings of the present study which may again reflect the enriched sampling of epithelial cells following collection with the cytobrush and the criteria used to define subclinical endometritis. Our observations may be important since high concentrations of TNF- α can prolong the estrous cycle in cattle by inducing PGE production which then contributes to the maintenance of progesterone production by the CL (Fischer et al., 2010; Okuda et al., 2010). These findings suggest that there may be an association between progesterone secretion and subclinical endometritis, which would provide a rationale for the use of prostaglandin F2- α as a therapy for subclinical endometritis (Galvão et al., 2009a).

Roach et al. (2002) reported that TNF- α is the main cytokine involved in the stimulation of the expression of adhesion molecules, such as E-Selection. Adhesion molecules play an essential role in the recruitment of PMNs following a response to IL-8. After bacterial invasion of the uterine lumen, endometrial cells and macrophages are stimulated to produce and release pro-inflammatory cytokines such as TNF- α and IL-6 which activate the expression of cell adhesion molecules by the vascular endothelium. Our findings also indicate that there was a significant correlation between IL-8 and TNF- α , IL-8 and IL-6 and IL-6 and TNF- α gene expression in cows with subclinical endometritis. Therefore, the present results suggest that analyzing a single chemokine, such as IL-8, may be sufficient to monitor uterine inflammation.

In conclusion, the cytobrush technique can provide sufficient uterine cells to perform both cytology and gene expression analysis with a single sample. The technique is non-invasive, and the samples obtained using a cytobrush are representative of endometrial cells which are directly involved in the uterine immune defense system. In the present study, the pro-inflammatory cytokines investigated reflect expression by

endometrial epithelial cells and possibly leukocytes recruited to the endometrial surface. Furthermore, the cytokine expression data supports using a threshold of >18% PMNs in endometrial cytobrush cytology for the diagnosis of subclinical endometritis when cows are examined between 28 and 41 days postpartum. Significantly elevated gene expression levels for IL-6, IL-8, and particularly TNF- α were present at 4 weeks after parturition, which is earlier than previously reported. Large increases in IL-6, IL-8, and TNF- α expression were associated with subclinical endometritis and likely play a key role in the pathogenesis of subclinical endometritis and possibly contribute to early embryonic losses in cows (Sheldon et al., 2009; Walsh et al., 2011).

The high correlation between PMNs in endometrial cytobrush cytology and IL-8 gene expression at 4 weeks postpartum indicates that monitoring IL-8 gene expression alone may be sufficient to predict uterine inflammation. Further investigations to clarify the impact of pro-inflammatory cytokines on reproductive performance in cattle are now possible with the development of a sensitive and non-invasive cytobrush sampling.

5. Endometritis-Positive Postpartum Dairy Cows: Ultrasonographic and Endometrial Cytobrush Features and Response to Benzathine Cephapirin Treatment

5.1. Abstract

Endometritis has been associated with poor reproductive performance in dairy cows. The objectives of the present study were to evaluate the effect of a single intrauterine infusion of benzathine cephapirin in endometritis-positive and endometritis-negative postpartum dairy cows at 28 to 41 days in milk (DIM); to compare ultrasonography and endometrial cytology for the diagnosis of endometritis in cows; and to determine the percentage of PMNs in endometrial cytology that reflected a benzathine cephapirin-responsive endometritis. A total of 340 lactating Holstein cows (96 primiparous and 244 multiparous) from 6 commercial dairy farms were enrolled. During scheduled herd visits, every 2 to 4 weeks, all postpartum cows between 28 and 41 days in milk (DIM) were examined using transrectal ultrasonography and endometrial cells were collected with the cytobrush method. The presence or absence of uterine fluid as reflected by uterine luminal diameter changes was recorded and where applicable, an assessment of uterine fluid volume was estimated by measuring the widest luminal diameter (cm). Endometrial and cervical thickness was also measured and the presence of ultrasonographically detectable structures on the ovaries (corpora lutea and follicles) was recorded. At that time, cows received at random an intrauterine infusion of benzathine cephapirin (Treated) or no treatment (Control). Cows assigned to the study were followed for reproductive data collection until 240 DIM. Based on the availability of 240-day pregnancy status, 262 of 340 cows (77%) between 28 to 41 DIM (34 ± 4.5 DIM) were included in the final analyses. Eleven of the 340 cows (3.2%) were diagnosed with clinical endometritis and were included with subclinical endometritis-positive cows in the study. The prevalence of endometritis in the current study was 18%. Endometritis-positive disease status was determined to be a threshold PMN percentage of $>15\%$ which was the lowest percentage of PMNs that was significantly associated with the positive response to benzathine cephapirin treatment. Treated cows with $>15\%$ PMNs required 31

fewer days ($P=0.041$) to become pregnant and had 2.5 times fewer services per conception ($P=0.0001$) compared to non-treated cows with $>15\%$ PMNs. Seventy-seven percent of cows with $>15\%$ PMNs in their endometrial cytospins also had a detectable corpus luteum, (OR: 2.3; $P=0.04$). The difference in uterine luminal diameter between cows with $>15\%$ and $<15\%$ PMNs was not significant ($P=0.28$). However, cows with measurable fluid in the uterine lumen became pregnant 33 days later than those without fluid ($P=0.001$). Treatment of cows with ultrasonographically detectable fluid with benzathine cephapirin had no effect on days open compared to treatment of cows without fluid in the uterus ($P=0.39$). Cervical diameter and endometrial thicknesses did not differ between groups of cows with high and low PMNs ($P=0.46$, $P=0.36$, respectively).

Based on the response, treatment of endometritis with benzathine cephapirin should be reserved for cows with $>15\%$ PMNs. Although ultrasonographic evidence of uterine fluid was associated with an increase in days open, it was not a positive indicator of cows with benzathine cephapirin-responsive endometritis.

5.2. Introduction

Postpartum bacterial contamination of the uterine environment has been reported to affect 80-100% of cows in intensively managed dairy herds. Although the immune system usually eliminates all of the offending microbes, persistent infection reportedly occurs in as many as 15 to 40% of postpartum cows (Sheldon et al., 2008).

Sheldon et al. (2006b) defined clinical endometritis as the presence of purulent uterine discharge detectable in the vagina 21 days or more postpartum or mucopurulent discharge detectable 26 days postpartum, and subclinical endometritis as inflammation limited to the endometrium at least 21 days postpartum with no detectable discharge from the vagina. Inflammation has been defined by the presence of $>18\%$ polymorphonuclear neutrophils (PMNs) in uterine cytospins prepared 21 to 33 days postpartum, or $>10\%$ PMNs in cytospins prepared 34 to 47 days postpartum (Kasimanickam et al., 2004). Although cows with subclinical endometritis do not have uterine discharge, impaired reproductive performance has been documented in a number of studies (Kasimanickam et al., 2005; Gilbert et al., 2005; LeBlanc, 2008; Barlund et al., 2008).

Barlund et al. (2008) reported that endometrial cytobrush cytology was the most reliable method of diagnosing subclinical endometritis in cattle. In that study, a number of diagnostic techniques were evaluated for their ability to predict pregnancy status at 150 days in milk (DIM) in cows sampled between 28 and 41 DIM. A threshold of $>8\%$ was the lowest proportion of PMNs associated with pregnancy status at 150 days. However, Barlund et al. (2008) reported that the low sensitivity (12.9%) and high specificity (89.9%) in detecting subclinical endometritis using endometrial cytobrush cytology indicated that most of cows that were pregnant by 150 DIM had $<8\%$ PMNs, whereas very few cows that were not pregnant at 150 DIM had $>8\%$ PMNs. This suggested that many other factors were likely involved, apart from the 8% PMN threshold, when a cow was not pregnant by 150 DIM.

Kasimanickam et al. (2004) considered the presence of any ultrasonographically detectable uterine fluid as evidence of subclinical endometritis. However, when compared with the most appropriate threshold proportion of PMNs for cows sampled using endometrial cytobrush cytology at 20-33 and 34-47 postpartum there was poor agreement ($\kappa = 0.28$). Barlund et al. (2008) found ultrasonographic measurement of a luminal diameter >1 mm was a more sensitive predictor of pregnancy status at 150 DIM than endometrial cytobrush cytology, but was less specific (Barlund et al., 2008). Using a threshold fluid measurement of >3 mm, the specificity for predicting pregnancy status at 150 DIM was the same as endometrial cytobrush cytology (PMN $>8\%$); however, the sensitivity was far less. Further analysis showed that there was little agreement between endometrial cytobrush cytology and ultrasonographically detected intrauterine fluid volume. Combining a threshold measurement of uterine luminal diameter of >3 mm with endometrial cytobrush cytology nearly doubled sensitivity while specificity only declined slightly. The conclusion was that there were likely two populations of cows with subclinical endometritis: those with a marked cellular response and little fluid accumulation and those with fluid accumulation and a modest or negligible detectable cellular response (Barlund et al., 2008). Barlund et al. (2008) recommended that researchers should employ both diagnostic techniques to improve accuracy; however, there remains a need to develop appropriate cow-side tests and to substantiate recommended diagnostic criteria based on real economic effects.

Benzathine cephapirin (Metricure®, Intervet Schering Plough, Canada, Ontario) has been reported to be the most effective antibiotic for intrauterine (IU) infusion in lactating dairy cows (LeBlanc et al., 2002b; McDougall et al., 2001b) and has been shown to be more effective than PGF2 α treatment for subclinical endometritis (Kasimanickam et al., 2005a). Kasimanickam et al. (2005a) also reported that cows that were categorized as negative for subclinical endometritis using endometrial cytobrush cytology also showed some improvement in reproductive performance following treatment with benzathine cephapirin, suggesting that these animals may have had endometrial disease. Therefore, analysis of the response to benzathine cephapirin therapy may represent a novel way to evaluate the criteria used to diagnose subclinical endometritis in postpartum dairy cows.

It has been reported that the presence of cervical diameter greater than 7.5 cm after 20 days postpartum in cows with clinical discharge was associated with endometritis (LeBlanc et al., 2002b). However, the majority of the cases in that study had clinical endometritis and the assessment of cervical diameter was subjectively estimated using transrectal palpation.

The objectives of the present study were: 1) to evaluate the effect of a single intra-uterine infusion of benzathine cephapirin in endometritis-positive (>8% PMNs) and endometritis-negative (\leq 8% PMNs) postpartum dairy cows 28 to 41 DIM, and to establish a threshold PMN percentage for endometritis based on the response to therapy; 2) to compare ultrasonographic features and endometrial cytology for the diagnosis of endometritis in cows. The unique feature of this study was the analysis and definition of endometritis under field conditions and the treatment-response to benzathine cephapirin using pregnancy status.

5.3. Materials and Methods

5.3.1. Animals and Study Design

A total of 340 lactating Holstein cows (96 primiparous and 244 multiparous) from six commercial dairy farms located near Saskatoon, Saskatchewan, Canada were

enrolled. The herds ranged in size from 75 to 150 milking cows and were housed in either tie-stall stanchion or free-stall barns. All herds used artificial insemination (AI) exclusively after a voluntary waiting period of approximately 55 days, range (45 to 60). All cows appeared healthy at the time of enrollment and had body condition scores of > 2.5 (scale 1 to 5). Cows were between 28 and 41 days in milk (DIM) at the time of initial examination and were identified prior to regularly scheduled herd visits. Cows were excluded from the study if they received systemic antibiotic or intrauterine therapy in the current lactation prior to enrolment. Data including days in milk at the time of sampling, parity, calving history and incidence of periparturient disease were recorded.

This project was approved by the University of Saskatchewan, University Committee on Animal Care and Supply.

5.3.2. Sampling and Examination

Prior to each farm visit an appropriate number of cytobrush units were assembled based on the number of cows to be sampled. Firstly, a stainless steel rod and stainless steel guard (50 cm long, 6 mm outside diameter) designed to cover the cytobrush and rod during passage through the vagina and cervix were removed from chemical sterilization media (CidexPlus®, Johnson & Johnson, Markham, Ontario) and rinsed with 20 ml sterile saline (Sodium Chloride 0.9%, Abbott Laboratories Ltd., Saint Laurent, Quebec). The rod was then placed inside the guard and using chemically sterilized (CidexPlus®) thumb forceps, a modified cytobrush was removed from its packaging and threaded into the rod. A plastic sheath (Chemise Sanitaires, IMV Technologies, France) was then placed over the assembled unit which was then placed inside a clean and sealable container for transport to the farm.

At each visit, eligible cows were restrained in either a head lock stanchion or chute. The perineum and vulva were cleansed with paper towels, and endometrial samples for cytological examination were collected using a modified non-sterile Cytobrush plus GT (Medscand Medical, Sweden; Barlund et al., 2008). Once cow-side, the assembled unit was retrieved and lubricant (Triad Sterile Lubricating Jelly, H&P Industries Inc., Mukwonago, WI, USA) was applied to the sheath prior to insertion into

the vagina. Next, a sleeved arm was introduced into the rectum to facilitate passage of the instrument through the cranial vagina and the cervix. At the external os of the cervix, the plastic sleeve was perforated and the guard and cytobrush were passed into and through the cervix. Once in the uterine body, the cytobrush was extended beyond the stainless steel guard and was rotated clockwise approximately 360 degrees to obtain cellular material from the adjacent endometrium. The cytobrush extension was then retracted into the stainless steel sheath to avoid contamination and then the stainless steel guard and cytobrush were removed from the reproductive tract. Cellular material was harvested by rolling the cytobrush onto a clean glass slide which was then allowed to air-dry on farm as described by Barlund et al. (2008).

Trans-rectal ultrasonography (Sonosite VET 180 Plus, Bothell, WA, USA) was used to evaluate: cervical diameter, the presence of uterine fluid and uterine luminal diameter, endometrial thickness and the presence of ovarian structures (corpora lutea and follicles). At the approximate mid-point of the cervix the greatest dorso-ventral diameter was identified and measured. Next, both uterine horns were examined for the presence of uterine fluid. If uterine fluid was detected, then the greatest luminal diameter containing fluid was identified and measured. The previously gravid horn, which was presumed to be the larger of the uterine horns, was scanned next and the area of greatest endometrial thickness was measured. All measurements were performed in triplicate and the average of these measurements was used for further analysis. Finally, both ovaries were examined and the presence of structures recorded.

Cows were randomly assigned to either treatment with benzathine cephapirin IU (Treated) or Control (no treatment) groups. During subsequent visits by the usual attending veterinarians, pregnancy diagnosis was performed between 35 and 60 days post-breeding using trans-rectal ultrasonography. For the duration of this study none of the cows were treated specifically for endometritis by the attending herd veterinarians; however, Ovsynch or Ovsynch plus progesterone-releasing device protocols were routinely employed in 2 of 6 herds for fixed-time AI. The other farms did not use any synchronization protocols in their herds. All assigned cows were followed for 240 days or until pregnancy was confirmed. All attending veterinarians were blinded to group designation (Treated or Control) and to the results of the endometrial cytobrush cytology

and ultrasonography. Appropriate reproductive data including pregnancy rate, interval from calving to pregnancy (days open), and service specific conception rate were obtained from Dairy Comp records for each herd until 240 days after parturition.

5.3.3. Cytology Examination

The air-dried slides were stained with modified Wright Giemsa stain, using an automated slide stainer (Hema-tek Slide Stainer; Miles Scientific, Naperville, IL, USA). All slides were examined using light microscopy at 400x magnification to identify individual cell types, including endometrial epithelial cells, and PMNs. Polymorphonuclear cell counts were expressed as the proportion PMNs counted out of combined PMNs plus endometrial cells. A total of 300 cells were counted by a single examiner. Cows were categorized as endometritis-positive or endometritis-negative on the basis of the percent PMNs/cytosmear.

5.3.4. Statistical Analyses

Various PMN percentage thresholds (>8 %, >11%, >14%, >15%, and >18%) were selected for further analysis. Reproductive performance data collected from each of the dairy farms was analyzed using STATA-10 (Stata, Version 10-0, StataCorp LP, Texas). Cox's proportional hazard regression and survival analysis were used to evaluate the effect of treatment on the hazard ratio of pregnancy and to determine the lowest PMN percentage significantly associated with days to pregnancy which would then be selected as the new percent PMN threshold.

Data for median days open in either four possible groups of cows treated (with < or >15% PMNs) or non-treated (with < or >15% PMNs) were analyzed using the Student's t-test. Cervical diameter and endometrial thickness data were analyzed using the Student's t-test to compare means between two groups of cows with high and low PMNs. Linear correlation coefficient regression analysis was applied to verify likely agreement on the uterine endometrial thickness, cervical diameter, and the percentage of PMNs. Agreement between each diagnostic test (endometrial thickness, cervical

diameter, and detectable uterine fluid) and the PMN percentage (>15%) was also evaluated using kappa. The number of services per conception was compared between treated and non-treated cows with >15% PMNs using the student's t-test. Likewise, the number of services per conception between treated and non-treated cows with <15%PMNs were compared using the student's t-test.

Survival curves were generated using Kaplan-Meier Analysis to evaluate the effect of treatment on cows diagnosed with ultrasonographically detectable fluid in the uterus, and parity. The odds ratio of having an ultrasonographically detectable CL at the time of examination between the group of cows with >15% and <15% PMNs was analyzed. Pearson's Chi-square test was used to compare parity with the presence of ultrasonographically detectable fluid in the uterus.

5.4. Results

The mean day of examination was 34 (± 4.5) DIM. Due to the availability of 240-day pregnancy status data, 262 of 340 cows were included in the final analysis. Seventy-eight (23%) cows enrolled in the study were removed due to culling, death, or not being bred prior to 240 days postpartum. Eleven of 340 (3.2%) cows had clinical endometritis diagnosed by the presence of mucopurulent or purulent exudate visible at the vulva in addition to an abundance of PMNs visible on the cytospin. Of 262 remaining cows, 154 (59%) received no treatment, and 108 (41%) were treated with benzathine cephapirin. Nine percent (31 of 340) of the cows had at least one of the following predisposing conditions: birth to twins, retained placenta, metritis, or displaced abomasums after calving.

In the group of treated cows, the lowest percentage of PMNs that was significantly associated with reduced days open (pregnancy status at 240 days postpartum) was >14% (Table 5.1), but because of the small difference between the number of cows with >14% PMNs (n=62) and >15% PMNs (n=58) in endometrial cytobrush cytology in this study, >15% PMNs was the lowest percentage of PMNs which was selected as the criteria to diagnose endometritis.

% PMNs	Number	Risk ratio	95% CI	P value
<i>Of cows</i>				
>8.0	73	1.34	0.83, 2.17	0.22
>11.0	69	1.03	0.54, 1.94	0.92
>14.0	62	1.85	1.02, 3.34	0.041
>15.0	58	1.85	1.02, 3.34	0.04
>18.0	54	1.80	0.99, 3.29	0.05

Table 5.1: Thresholds for percentage of polymorphonuclear cells (PMNs) on endometrial cytobrush cytology evaluation of 262 cows inseminated at least once using Cox's proportion hazards model adjusted for farm and days in milk at sampling.

Using a threshold >15% PMNs, the prevalence of endometritis in the current study was 18% (n=340) with an individual herd prevalence ranging from 14 to 33%. The 11 cows with clinical disease were included in the population of cows with >15% PMNs for further analysis. The proportion of cows with at least one of the conditions: retained placenta, metritis, displaced abomasums, or twins during the current lactation and a PMN percentage >15% was 84.6%, 100%, 33.4%, 71.4%, respectively.

Endometritis-positive cows treated with benzathine cephapirin became pregnant 31 days earlier than non-treated endometritis-positive cows ($P=0.041$). The median days open for cows diagnosed with endometritis (>15% PMN) and treated with benzathine cephapirin was 118 days compared to 149 days for non-treated cows. The effect of treatment on time to pregnancy in endometritis-positive cows is demonstrated using survival analysis (Fig 5.1). The median days open for non-treated cows with endometritis was 149 days compared to 89 days for endometritis-negative cows (<15% PMNs; $P=0.007$). Non-treated, endometritis negative cows required 60 fewer days to become pregnant than their non-treated counterparts with endometritis ($P=0.007$) (Fig 5.2). The median days open for treated endometritis-negative cows was 108 compared to 89 days for non-treated endometritis-negative cows ($P=0.91$).

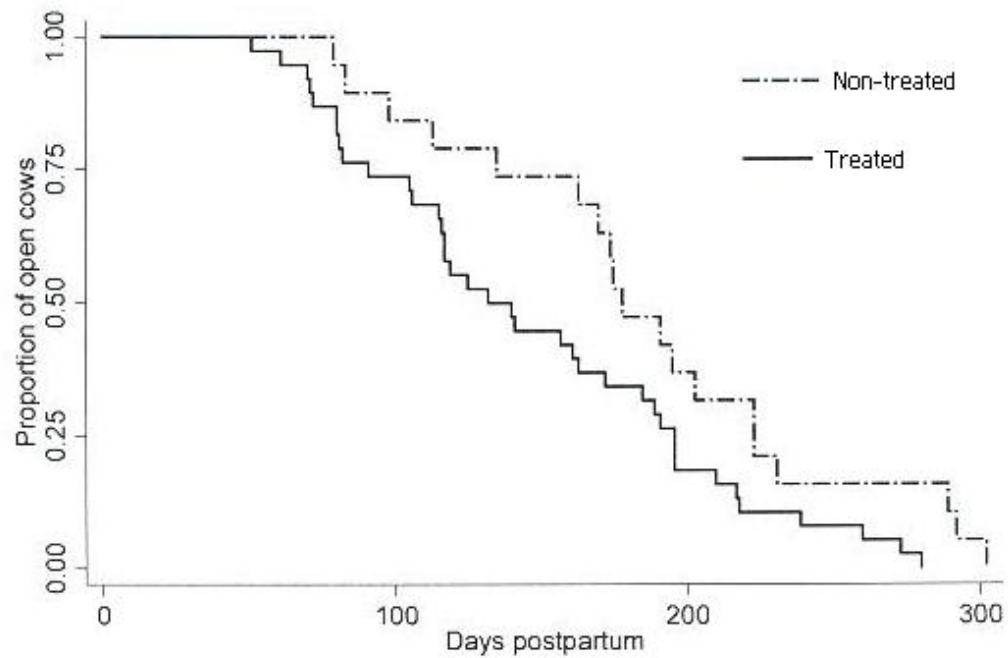


Figure 5.1: Survival analysis: the association of treatment with benzathine cephalixin on interval to pregnancy in dairy cows diagnosed as endometritis-positive by endometrial cytobrush cytology (threshold >15% PMNs) (n=58) ($P=0.04$).

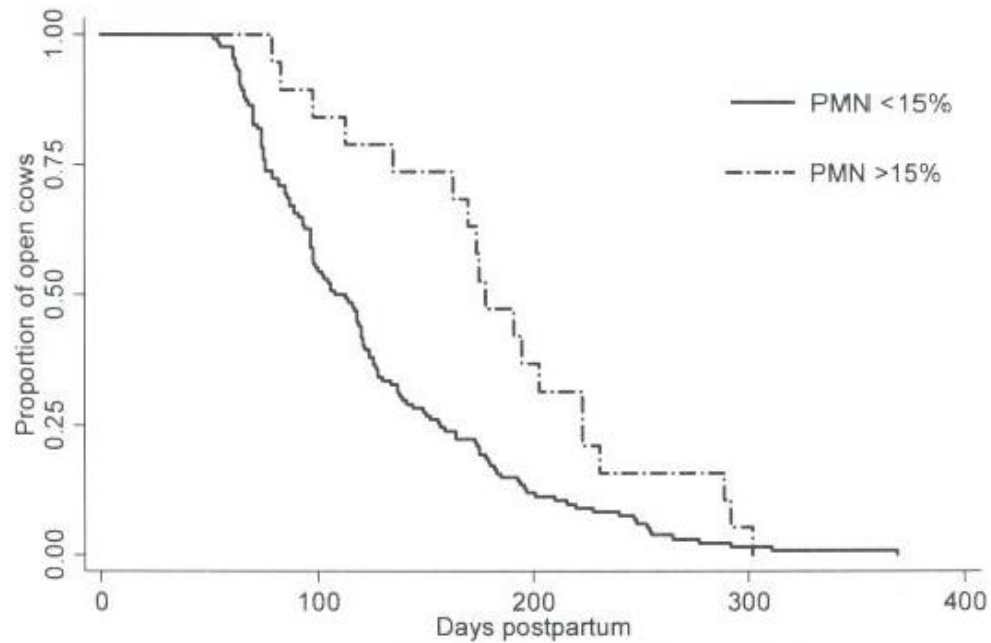


Figure 5.2: Survival analysis: the association of endometritis, diagnosed by endometrial cytobrush cytology (threshold >15% PMNs) between 28 and 41 days in milk postpartum, on interval to pregnancy in non-treated cows (n=154) ($P=0.007$).

In this study, 98 of 340 cows (28%) had ultrasonographically detectable uterine fluid with a mean luminal diameter of $0.73 (\pm 0.85)$ cm. Of those, 35 cows (36%) had a PMN percentage >15% whereas, 63 cows (64%) had a PMN percentage <15%. The mean uterine luminal diameter in cows with >15% and <15% PMN percentages was 0.85 ± 0.87 cm and 0.65 ± 0.83 cm, respectively ($P=0.28$). Furthermore, the correlation between cows with evidence of uterine fluid and endometritis based on a threshold proportion of PMNs >15% on cytobrush cytology was not significant ($P=0.08$, coefficient of determination R^2 was 0.027). When all cows, regardless of treatment categories, were included in a

survival analysis, median days open for cows with uterine fluid was 141 days compared to 108 days for cows without uterine fluid ($P=0.001$) (Fig 5.3).

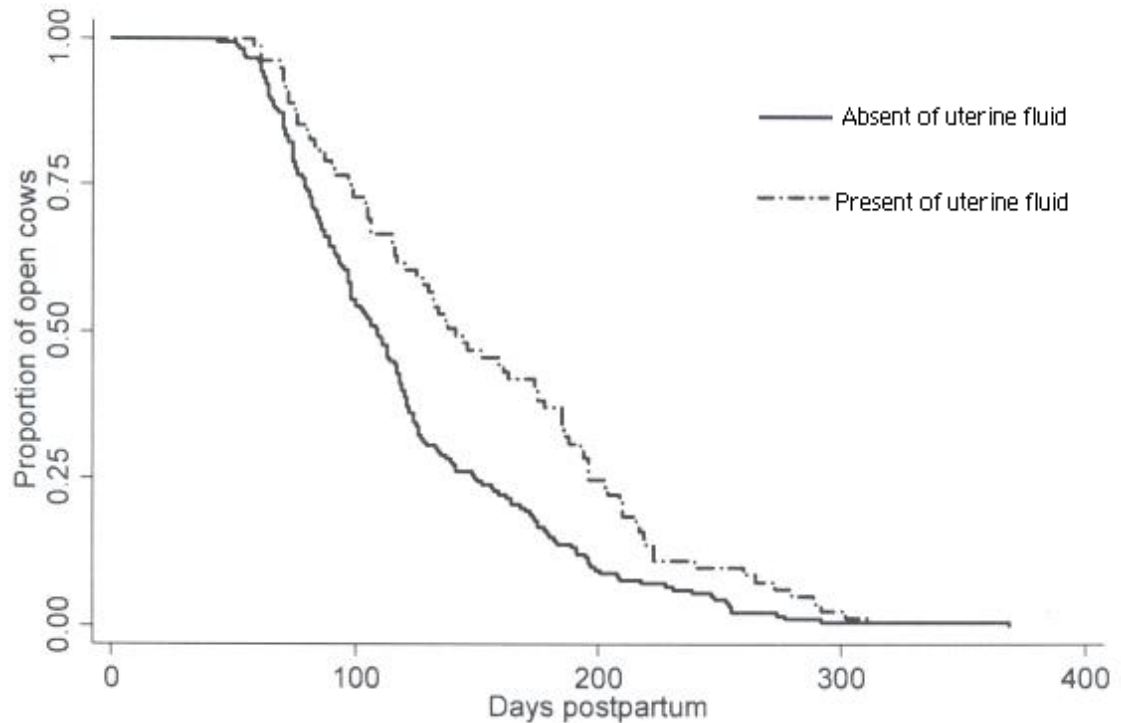


Figure 5.3: Survival analysis: the association of endometritis diagnosed by detectable fluid in the uterus between 28 and 41 days postpartum on interval to pregnancy in dairy cows ($n=262$) ($P=0.001$).

Benzathine cephapirin treatment had no effect on the interval to pregnancy in cows with uterine fluid ($P=0.39$). When the effect of parity on fluid accumulation was analyzed, a greater number of multiparous cows ($n=79$) had uterine fluid than their primiparous counterparts ($n=19$) ($P=0.04$).

The mean cervical diameter in the group of cows with $<15\%$ PMNs was 3.8 ± 0.4 whereas, the mean for cows with $>15\%$ PMNs was 3.9 ± 0.5 ($P=0.36$). The correlation between cervical diameter and PMN percentages $>15\%$ and $<15\%$ was not significant ($P=0.46$, Coefficient of determination R^2 was 0.0016). Similarly, there was no difference

in endometrial thickness between the >15% PMN (0.7 ± 0.1 cm) and <15% PMN groups (0.7 ± 0.1 cm; $P=0.89$) and no significant correlation between endometrial thickness and PMN percentage ($P=0.53$, coefficient of determination R^2 was 0.021) was observed.

Agreement between a threshold of >15% PMNs as the reference technique and endometrial thickness, cervical diameter, and detectable uterine fluid were evaluated using kappa. There was no agreement between endometrial thickness and cervical diameter with PMN percentages >15% whereas, there was poor agreement between uterine fluid and PMN percentages >15% (Table 5.2).

Diagnostic Technique	Kappa	(P Value)
Cervical thickness	-0.0007	(0.68)
Uterine fluid	0.0081	(0.3)
Endometrial thickness	-0.0004	(0.64)

Table 5.2: Evaluation of agreement using Kappa between a polymorphonuclear cell percentage >15, and ultrasonographic detection of: cervical diameter, uterine fluid, and endometrial thickness measurements in cows.

The proportion of cows with CLs at the time of examination via ultrasound was investigated. Two hundred and twenty-two of 340 cows in this study had CL(s) at the time of examination. A greater proportion (77%) of cows (45 of 58) with endometritis (PMNs > 15%) had a detectable CL at the time of examination, compared to 63% of cows (177 of 282) with <15%. Therefore, the odds of an individual having a detectable CL was 2.3 ($P=0.04$) in the group of cows with >15% PMNs.

Overall, the mean number of services per conception for the endometritis-positive cows treated with benzathine cephapirin was 1.8 versus 4.3 for non-treated endometritis-positive cows ($P=0.0001$). However, the mean number of services per conception for endometritis-negative cows that were treated with benzathine cephapirin (2.2 services per conception) did not differ from that of non-treated endometritis-negative cows (2.3 services per conception; $P=0.71$). Therefore, treated endometritis-positive cows required

2.5 fewer services per conception compared to non-treated endometritis-positive cows ($P=0.00015$). However, the mean number of services per conception in the group of treated cows diagnosed as endometritis-positive versus endometritis-negative did not differ (1.8 versus 2.2, $P=0.38$). In non-treated cows with $<$ and $>15\%$ PMNs, there was a difference between the mean number of services per conception, 4.3 and 2.3, respectively ($P=0.0001$). The first service conception rate for benzathine cephapirin treated endometritis-positive cows was 38% versus 5.3% for the non-treated endometritis-positive cows ($P=0.03$). The first service conception rate for benzathine cephapirin treated endometritis-negative cows was 35% compared to 64% for the non-treated endometritis-negative cows ($P=0.08$).

Cow parity in the current study ranged from 1 to 10 with a median parity of 2.0. Fifteen of 96 primiparous cows (15.6%) had PMN proportions $>15\%$ whereas, 50 of 244 (20%) of multiparous cows had PMN proportions $>15\%$ ($P=0.56$). Primiparous cows treated with benzathine cephapirin had a median days open of 113 compared to 139 (difference – 26, $P=0.005$) for multiparous treated cows (Fig 5.4).

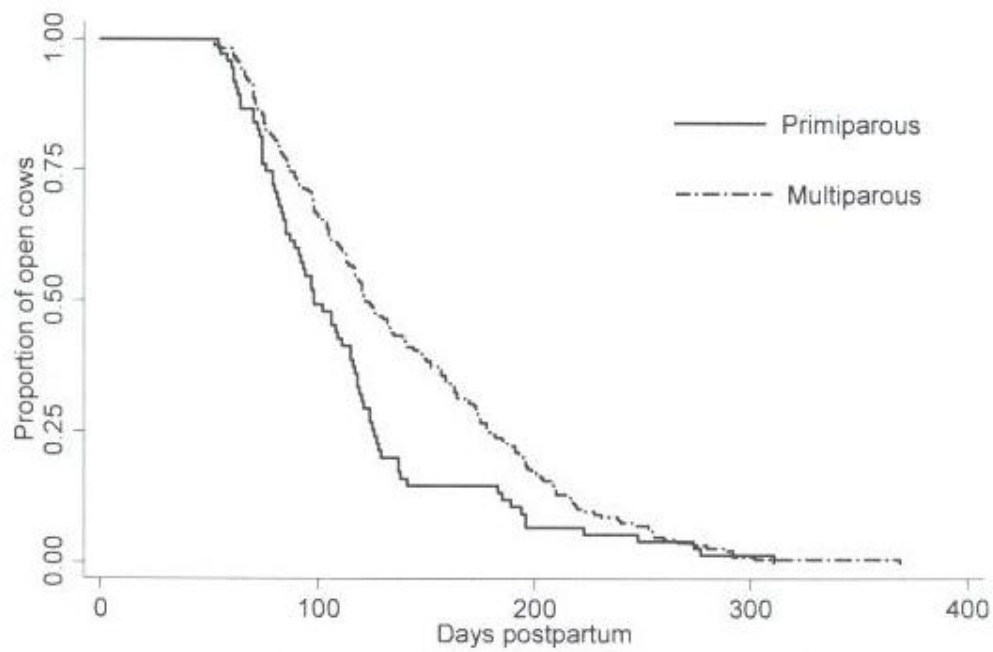


Figure 5.4: Survival analysis: the effect of treatment on relative pregnancy rate for benzathine cephapirin between 28 and 41 DIM based on primiparous versus multiparous cows (n=262) ($P=0.005$).

Non-treated endometritis-negative primiparous cows had a median days open of 108 compared to 119 for non-treated multiparous cows (difference – 11, $P=0.77$).

5.5. Discussion

Various percentages of PMNs in the endometrial cytology samples(>8%, >11%, >14%, >15%, and >18%) were evaluated to determine the most appropriate threshold for the diagnoses of endometritis based on response to a single uterine infusion of 500 mg of benzathine cephapirin in dairy cows between 28 and 41 days postpartum. A threshold of >15% PMNs was found to be the most appropriate. In a related study, Ghasemi et al. (2011; submitted and included in this thesis) showed that a threshold of >18% was the lowest PMN percentage which was significantly associated with an elevation of three endometrial pro-inflammatory cytokines, IL-6, IL-8 and TNF- α in cows sampled between 28 and 41 days postpartum. These threshold PMN percentages are greater than what has been reported previously where the most appropriate threshold was >8% PMNs for defining endometritis-positive disease status in cows sampled between 28 and 41 (Barlund et al., 2008), and 25 (Dourety et al., 2011) days postpartum using 150 and 270 day pregnancy status as the outcome, respectively. Kasimanickam et al. (2004) reported that a threshold of >18% PMNs was the most appropriate for cows examined between 20 and 33 DIM, and that >10% should be used for cows examined between 34 and 47 DIM. Although the DIM at sampling ranged between 28 and 41 in the current study, the mean was 34 (± 4.5) DIM. Support for a higher threshold is justifiable based on the results of the present study as it represents benzathine cephapirin-sensitive bacterial infection in the uterus rather than the PMN influx associated with the normal involution process.

The unique feature of the current study was the analysis and definition of endometritis under field conditions based on the effect of treatment with benzathine cephapirin on days open rather than simply determining a threshold PMN percentage based on differences in days open as used in other studies (Kasimanickam et al., 2004; Barlund et al., 2008). Investigators using pregnancy outcome alone to determine an appropriate threshold proportion of PMNs for the diagnosis of endometritis have noted that there are numerous factors which can influence pregnancy status (Barlund et al., 2008) which will reduce the accuracy of the recommended PMN threshold. A basic and arguably inappropriate assumption, in the current study, was that all cases of

endometritis would respond to benzathine cephapirin treatment. Few treatments are 100% effective, but support for this approach is provided by the fact that there was no difference in the median days open between treated and non-treated cows with < 15% PMNs, whereas treatment of cows with >15% PMNs resulted in a 31-day improvement in days open as compared to non-treated controls. Furthermore, based on the findings of the current study support for a higher PMN threshold is justifiable because of no difference in the number of days open between the treated and non-treated cows when the PMN thresholds of >8% and >11% were applied. This lack of an apparent difference was probably due to a large number of cows spontaneously recovering or more precisely, several cows had elevated PMN percentages associated with normal uterine involution. While the PMN threshold of >14% was statistically significant in the current study, there were too few cows within the >14% and >15% PMNs treatment groups to determine if the differences were real or due to chance (Table 5.1). Therefore, we decided to use the more conservative value of >15% PMN to prevent the needless treatment of healthy cows.

There have been a few controlled clinical trials, investigating the effects of antibiotic treatment on the reproductive performance of cows with uterine abnormalities (Gustafsson et al., 1984; Paisley et al., 1986; Steffan et al., 1984; Kasimanickam et al., 2005a). In the present study, treatment of cows with endometritis resulted in those animals becoming pregnant 31 days earlier than non-treated endometritis-positive cows. Kasimanickam et al. (2005a) also showed that median days open for the cows that were treated with either benzathine cephapirin or cloprostenol was 26 days shorter than non-treated controls; however, in the current study, endometritis-negative cows (PMNs<15%) did not benefit from intrauterine treatment. Other investigators have reported that cows which were diagnosed as endometritis-negative (LeBlanc et al. 2002b) or subclinical endometritis-negative (Kasimanickam et al., 2005a), and treated with benzathine cephapirin had 11 and 26 fewer median days open, respectively than their non-treated counterparts. Therefore, it may be speculated that many of the cows diagnosed as endometritis or subclinical endometritis-negative in these studies actually had the disease i.e., false negative.

In the current study, non-treated cows with endometritis became pregnant 60 days later than non-treated endometritis-negative cows. Barlund et al. (2008) showed that cows with cytological evidence of endometritis (PMNs >8%) became pregnant 24 days later than non-diseased (PMNs<8%) cows, which was much less than the findings in the current study and the 88-day difference reported by Gilbert et al. (2005). The apparent discrepancy between Barlund et al. (2008) and the current study, both examining cows between 28 and 41 DIM may be explained by the different PMN thresholds used in these experiments; whereas, Gilbert et al. (2005) used a threshold of 5% and examined cows between 40 and 60 DIM.

Bacterial growth and impaired uterine involution have been shown to be associated with ultrasonographically detectable intrauterine fluid accumulation (Mateus et al., 2002). Dourey et al. (2011) reported that cows with higher quantities of fluid also had higher PMN percentages in their endometrial cytobrush cytologies. The results of the present study failed to show a relationship between the accumulation of uterine luminal fluid and PMN percentages. Barlund et al. (2008) and Kasimanickam et al. (2004) also reported that there was poor agreement between the ultrasonographic measurement of luminal fluid and the PMN threshold used in their respective studies to identify cows with endometritis. Both Kasimanickam et al. (2004) and Barlund et al. (2008) concluded that there were two populations of cows with endometritis: cows that had no fluid accumulation, but a cellular response to inflammation (PMNs), and cows that have fluid accumulation and a low concentration of inflammatory cells, possibly associated with impaired or decreased uterine clearance (Palmer, 2008; Barlund et al., 2008). Therefore, findings in the current study lend support to the theory of the existence of two populations of cows with endometritis: cows that have a cellular inflammatory response with no fluid accumulation, and cows that have impaired uterine clearance with fluid accumulation and less cellular response to inflammation.

In the present study, cows with ultrasonographically detectable luminal fluid did not benefit from benzathine cephapirin administration. Based on this finding we speculate that the presence of fluid in the uterus may be associated more with an impaired uterine clearance in this group of cows. Factors such as the position of the uterus in the pelvis or abdomen or decreased uterine contractility rather than a primary bacterial infection may

be more of the cause. Another theory is that fluid or debris in the uterine lumen may somehow dilute or inactivate benzathine cephapirin rendering it ineffective. Still other possibilities could be the presence of organisms, or biofilms in the uterus which may exhibit benzathine cephapirin resistance. These findings are likely the reason for the poor agreement between luminal fluid accumulation measurements and the cytobrush cytology-derived PMN threshold reported by Barlund et al. (2008). Cows with fluid accumulation in the uterus might benefit from treatment with PGF_{2α} to improve uterine clearance mechanisms, or may require more than one treatment with benzathine cephapirin.

Seventy-seven percent of cows with >15% PMNs had a CL, which could have contributed to the development of endometritis. The odds ratio of a cow in the group of cows with >15% PMNs having a CL was 2.3. There have been reports that prolonged luteal phases are caused by uterine abnormalities and the inability of the uterus to produce prostaglandins (Farin et al., 1989; Opsomer et al., 1998; Walsh et al., 2011). Lewis et al. (1997) reported that the occurrence of uterine infections was reduced under the influence of estrogen, and there is a confirmed association between progesterone concentrations and the development of endometritis (Galvão et al., 2009a). Therefore, using an appropriately spanned combination of prostaglandin therapy and an IU infusion with 500 mg benzathine cephapirin in cows diagnosed with endometritis and with a palpable CL might improve reproductive performance.

The findings in the present study indicate a negative effect of parity on response to treatment with benzathine cephapirin in cows with >15% PMNs. Disease-positive primiparous cows became pregnant 26 days sooner than multiparous disease-positive cows following treatment with 500 mg benzathine cephapirin. Also related to this observation was the finding that multiparous cows accumulated significantly more fluid in the uterus than primiparous cows. This would suggest that the decreased effect of treatment with benzathine cephapirin in multiparous postpartum cows was associated with a breakdown in uterine clearance mechanisms. Cows with increased susceptibility to persistent endometritis may have impaired myometrial contractility in response to acute inflammation resulting in an accumulation of fluid and inflammatory products within the uterine lumen as has been documented in mares (Troedsson, 1999). Therefore, in order to

have a greater understanding of uterine diseases and to develop more effective treatment regimes, uterine disease studies focusing on bacterial infection as a major cause of impaired reproductive performance in cows need to be expanded toward increasing our understanding of the causative mechanisms. Future studies may show that cows with ultrasonographic evidence of fluid accumulation or multiparous cows diagnosed with endometritis may also benefit from prostaglandin administration or a second treatment with benzathine cephapirin.

The current study has established that the cervical diameter and endometrial thicknesses did not differ significantly between groups of cows with high and low percentage of PMNs ($>$ or $<15\%$) in endometrial cytology. It has been reported that a cervical diameter of greater than 7.5 cm after 20 days postpartum was associated with clinical endometritis in cows (LeBlanc et al., 2002b). In the current study, there were only 11 cows with clinical endometritis versus 47 with only subclinical endometritis. Barlund et al. (2008) reported that the use of ultrasonographic measurement of endometrial thickness as a diagnostic test was not a sensitive indicator of pregnancy status at 150 days. The findings in the current study support the notion that ultrasonographic measurement of both cervical diameter and endometrial thickness is not useful for detecting cows with endometritis.

In conclusion, findings in this study indicate that: 1) benzathine cephapirin is a very effective treatment for endometritis in the postpartum dairy cow; 2) endometrial cytobrush cytology is a more sensitive and useful diagnostic tool than ultrasonography to diagnose endometritis in postpartum dairy cows; and 3) that the threshold value of $>15\%$ PMNs in endometrial cytobrush cytologies should be used as a criterion for treatment with intrauterine benzathine cephapirin in postpartum cows 28 to 41 DIM.

6. General Discussion

The central theme of this thesis was improving our ability to accurately diagnose endometritis in postpartum dairy cattle using endometrial cytology. This was achieved by evaluating the treatment-response to benzathine cephapirin and investigating the association between endometritis and pro-inflammatory cytokine gene expression to determine a new and more appropriate percentage PMN diagnostic threshold.

A primary production goal in postpartum dairy cow management is to establish pregnancy in an efficient manner and at a profitable interval after calving. While the fertilization rates in cows may be as high as 90%, conception rates are lower (Pomalar et al., 2005). The most common reasons for reproductive failure in high producing dairy cows are problems in the detection of estrus and early embryonic losses (Humboldt, 2001). Nebel (1999) stated that good estrus detection, high quality semen, good insemination technique and a healthy uterine environment are essential for high reproductive performance in dairy cows. A high prevalence of uterine disease such as endometritis dramatically impairs the reproductive performance of high yielding dairy cows due to persistent bacterial infection which leads to inflammation and damage to the endometrium thereby, prolonging uterine involution and impairing fertility (Fourichon et al., 2000; Gilbert et al., 2002; Kasimanickam et al., 2004).

We planned two studies. First a field study to evaluate a relatively new and effective therapy, intrauterine benzathine cephapirin, to define postpartum endometritis; and secondly to determine the relationship between endometrial PMN proportions and pro-inflammatory cytokines. To accomplish our task, we utilized the modified cytobrush technique to harvest endometrial cells to determine the PMN percentage, and to provide sufficient endometrial cellular material for mRNA isolation. We evaluated the association between selected pro-inflammatory cytokine gene expression (IL-6, IL-8, TNF- α) and the PMN percentage in endometrial tissue and the correlation between pro-inflammatory cytokine expression and PMN percentage with regard to the case definition for postpartum subclinical endometritis. Furthermore, we evaluated the relationship between pro-inflammatory cytokines because pro-inflammatory cytokines such as TNF- α may

induce the production of IL-8 which recruits PMNs to sites of inflammation (Roach et al., 2002).

Bacterial contamination of the uterus around calving has been well documented, but typically the natural clearance mechanisms of the uterus eliminate bacterial infection and consequent inflammation within the first 3 to 4 weeks after parturition. Walsh et al. (2011) reported greater uterine inflammation in cows that developed persistent (chronic) bacterial endometritis. Invading bacteria activate the secretion of pro-inflammatory cytokines and the production of chemokines which lead to PMN recruitment into the endometrium. Therefore, cows that develop endometritis have an increased influx of PMNs into the uterine lumen around the fourth week postpartum (Barlund et al., 2008). Cytokines, which are the main products of the inflammation cascade, are also reported to be involved in several developmental processes during embryogenesis (Zerbe, et al 2003). Embryonic implantation depends on a receptive and healthy uterine environment. Communication between the embryo and the uterus occurs through cytokines and growth factors produced by the endometrium, some of which (IL-1) also act as inflammatory mediators in endometritis (Kauma, 2000). An imbalance in cytokine secretion, such as inappropriate, ill-timed or unregulated secretion may contribute to persistent inflammation and lead to endometritis, failure to conceive or early embryonic loss, as has been reported in the mare (Fumoso et al., 2003); however, these problems are yet to be investigated in the cow.

In the current study, >18% PMNs on endometrial cytobrush cytology was the lowest percentage of PMNs associated significantly with an elevation of the three pro-inflammatory cytokines measured, IL-6, IL-8 and TNF- α . In fact, a >15% PMN threshold was correlated significantly with IL-8; however, the small number of cows in the current study limited the statistical power. A clear understanding of these relationships may be addressed using a larger sample size. Another limitation of the current study was that there were no bacterial culture results to aid in the interpretation of the endometrial cytobrush cytology samples. Without culture results, the cause of the elevated cytokine mRNA levels associated with >18% PMNs in the endometrial cytobrush cytology could not be definitively identified. Furthermore, culture results could have provided a stronger link between the pro-inflammatory cytokine and the

benzathine cephapirin studies. Postpartum uterine cytokine mRNA expression levels may be influenced by the time postpartum, the degree of uterine involution, the presence of pathogenic bacteria, or both together.

The findings in our study also provided evidence that there was a direct link between the percentage of PMNs in endometrial cytobrush cytology and the levels of selected endometrial pro-inflammatory cytokine gene expression (IL-6, IL-8 and TNF- α) in endometritis-positive cows. Our results agreed with those of Fischer et al. (2010) who reported that the pro-inflammatory cytokines, IL-6, IL-8 and TNF- α played a major role in the influx of PMNs into the uterus. The most substantial result in the cytokine study reported herein was the highly significant positive correlation ($P = 0.0001$) between IL-8 levels and the PMNs threshold of $>18\%$. This supported our hypothesis that there is an association between the expression of endometrial cytokines and cytobrush PMN counts. Furthermore, it can be concluded that IL-8 expression provides strong validation for the use of a PMN threshold of $>18\%$ for the diagnosis of endometritis in the cow at this stage in the postpartum interval (28 to 41 DIM). This result is consistent with the findings of Zerbe et al. (2003) who showed that an infusion of human recombinant interleukin-8 (rhIL-8) into the uterus of mares and cows resulted in PMN recruitment. Collectively, these results suggest that IL-8 plays a causative role in acute inflammation in the uterus by recruiting and activating PMNs. The data from the current study and Zerbe et al. (2003) also suggest that IL-8 is biologically relevant and is the main cause of PMN recruitment. Compared to the other cytokines evaluated, the expression levels of IL-8 may most accurately reflect the inflammation status of the cow's uterus. Furthermore, Zerbe et al. (2003) showed that anti-IL-8 treatment prevented infiltration of PMNs and PMN dependent tissue damage. Studies directed at evaluating the regulation of IL-8 driven PMN recruitment in the bovine uterus may suggest a new direction in the treatment of postpartum endometritis.

Researchers have used various PMN thresholds and subsequent pregnancy status as an outcome to define endometritis in dairy cows; however, pregnancy status is influenced by numerous variables such as nutrition, genetics, body condition, metabolic disorders and uterine pathology (Barlund et al., 2008; Walsh et al., 2011) making it difficult to identify a gold standard for the diagnosis of endometritis. Pregnancy status

may not be the best outcome to use as a determinate of the potential reproductive performance of postpartum dairy cows, particularly when management is sub-optimal or variable. Diagnosis of endometritis in cows; however, still remains one of the greatest challenges for practitioners (Kasimanickam et al., 2004). The response to benzathine cephapirin therapy was used in our study as a unique feature to evaluate and analyze the criteria used to diagnose postpartum endometritis under field conditions and in relation to days to pregnancy rather than simply comparing pregnancy outcomes. Treatment with benzathine cephapirin was chosen as a means to identify an appropriate percentage PMN threshold because it was hypothesized that most bacterial infections would respond to antibiotic therapy, while an elevated percentage of PMNs associated with the normal uterine involution may not. Potential problems with these assumptions include that there may be bacteria present in the uterus that are resistant to the benzathine cephapirin, the single dose therapy may not be effective, or the vehicle alone may cause a response. We did not have sham treated controls in this study.

In the benzathine cephapirin experiment (chapter 5), >15% PMNs was the lowest mean percentage of PMNs that was significantly associated with the positive effect of treatment with benzathine cephapirin to pregnancy in dairy cows. Cows treated at 28 to 41 DIM with endometritis (PMNs >15%), as defined in the current study became pregnant 31 days earlier than non-treated cows with >15% PMNs on endometrial cytobrush cytology. This only partially supports our second hypothesis that benzathine cephapirin improves reproductive performance in dairy cows. However, endometritis-negative cows (PMNs <15%) did not benefit from intrauterine treatment, while non-treated cows with high PMNs (>15%) in their endometrial cytobrush cytology became pregnant 60 days later than their herd mates with low PMNs (<15%). These findings also support our second hypothesis in that the response to benzathine cephapirin treatment allowed us to establish a new threshold proportion of PMNs (>15%) based on the response to treatment.

It should be kept in mind that the response to treatment is dependent on the PMN threshold applied to define endometritis and the time after parturition when the cytobrush sample is taken. For example, Kasimanickam et al. (2005) reported that cows which were diagnosed as endometritis-negative and treated with benzathine cephapirin had a median

days open of 26 days less than the untreated control group. In that particular study, the presence of >18% PMNs in endometrial cytobrush cytology was the diagnostic criterion used to define endometritis in dairy cows between 20 and 33 DIM. However, it should be taken into account that many cows spontaneously eliminate the bacterial contamination during the first 4 weeks after parturition. In the study by Kasimanickam et al. (2005), the cows which received the treatment (benzathine cephapirin) were between 20 and 33 days DIM which is earlier than in our study (34 ± 4.5 DIM). Although the authors did not indicate the mean or median days postpartum, it is likely that several recovered spontaneously prior to breeding. It is likely that cows in their study which had normal levels of bacterial contamination were also included in the endometritis-positive group. If these same cows were evaluated a week later it might have been shown that they spontaneously resolved. Recommendations based on these early postpartum cows would, therefore, result in the treatment of more cows than is necessary.

Mateus et al. (2002) reported that bacterial growth and impaired uterine involution have been associated with ultrasonographically detectable intrauterine fluid accumulation. Ultrasonography is a practical cow-side test that could potentially be used to identify diseased cows; however, in the present study, a significant relationship between detectable luminal fluid and percentage PMNs was not identified, in agreement with Barlund et al. (2008). Kasimanickam et al. (2004) also reported that there was poor agreement between the ultrasonographic measurement of luminal fluid, and cytological diagnostic test results to identify cows with endometritis. These findings have led to both groups concluding that there were likely two populations of cows with endometritis: cows that have no fluid accumulation, but a cellular response to inflammation (PMNs), and cows that have fluid accumulation and a decreased concentration of inflammatory cells possibly associated with impaired or decreased uterine clearance (Kasimanickam et al., 2004; Barlund et al., 2008). Therefore, findings in the current study support the existing theory of the two populations of cows with endometritis as was reported in previous studies.

Ultrasonographic measurement of the cervical diameter or endometrial thickness as a diagnostic test to identify endometritis in postpartum cows was evaluated in the present study, and a significant relationship between cervical diameter or endometrial

thickness and the percentage of PMNs in endometrial cytology was not found. This lack of sensitivity should be kept in mind when veterinarians utilize transrectal palpation and/or ultrasonography as part of their routine examination of postpartum cows. Furthermore, cows in the present study with ultrasonographically detectable luminal fluid did not benefit from benzathine cephapirin administration. Therefore, it can be speculated that the fluid accumulation in the uterus may be associated more with an impaired uterine clearance in this group of cows than with bacterial infection. In the current study, multiparous cows had a greater tendency to accumulate fluid in their uterus than primiparous cows. This finding again supports the conclusion that the fluid accumulation in the uterus might be considered as an indicator for impaired uterine clearance than bacterial infection. The reason for no benefit of the use of benzathine cephapirin in these cows is not clear, but one could speculate that the fluid and debris left in the uterus may possibly inactivate or interfere with the activity of benzathine cephapirin. Postpartum cows with uterine fluid accumulation might benefit from prostaglandin treatment because it is likely to induce estrus, which in effect softens and opens the cervix and increases uterine contractility, or more than one treatment with benzathine cephapirin is necessary to overcome the effects of fluid accumulation. In conclusion, while ultrasonography provides some information relating to the status of uterine clearance in cows, it provides little information on the detection of endometritis per se. Collectively, the results did not support our hypothesis that ultrasonography is a useful tool for the diagnosis of endometritis.

It has been reported that there is an association between the occurrence of endometritis and elevated circulating progesterone concentrations in postpartum dairy cows (Sheldon et al., 2004b). Postpartum ovarian activity is dependent upon the uterine immune response. Prolonged luteal phases in cows with endometritis may be caused by uterine abnormalities and the inability of the uterus to produce $\text{PGF2}\alpha$ (Lewis et al 1997; Galvão et al., 2009a). In the present study, 77% of cows with >15% PMNs had a CL, and the likelihood of having a CL was 2.3 times in cows with >15% PMNs versus those with <15% PMNs. It appears that cows with a CL are at increased risk of experiencing endometritis.

In summary, a case definition of >15% PMNs should be used as the threshold to define endometritis in lactating dairy cows that are between 28 and 41 DIM. This PMN threshold is supported by treatment-response to benzathine cephapirin in endometritis-positive cows. The small number of cows in the first experiment (Chapter 4) with high percentage PMNs (>18%) makes it difficult to draw a clear conclusion to select that PMN proportion as a definition for endometritis. Furthermore, the relationship between IL-8 expression and the >15% PMN threshold tended towards significance ($P = 0.056$). Cows with endometritis as defined in this thesis benefit from an IU infusion of benzathine cephapirin at 28 to 41 DIM, to improve reproductive performance. The results of this study could be used to more accurately diagnose endometritis and if cytobrush cytology is not already being used veterinarians should be encouraged to utilize cytobrush cytology when examining postpartum cows. Furthermore, uterine treatment of postpartum cows should be considered with regard to the cytobrush cytology results.

7. Suggestions for Future Research

The studies reported in this thesis answered the questions related to the three components of the general hypothesis. The relatively small numbers of cows with high PMNs (>18%) in the current study makes it difficult to draw conclusions about the optimal mean PMN threshold to use as the gold standard to define endometritis based on the relationship to pro-inflammatory mRNA cytokine levels in postpartum cows. Perhaps this portion this study could be repeated with more cows. The lack of bacterial culture results was identified as a limitation in the current study. My view is that the combination of culture results and pro-inflammatory mRNA cytokine levels would provide more complete answers to differentiate between pathological bacterial infections of the uterus versus normal pro-inflammatory cytokine gene expression associated with uterine involution in postpartum cows.

Further studies into the role of cytokines in the fertility of dairy cows and the development of techniques to accurately evaluate the uterine status in postpartum cows are needed in order to better understand this aspect of sub-fertility in cows at the uterine level. Further investigations into the ability of inexpensive and minimally invasive techniques such as endometrial cytobrush cytology and evaluation of pro-inflammatory cytokines before and after a single treatment with IU benzathine cephapirin may also contribute to our understanding of the effect of antibiotic treatment at the molecular level rather than using pregnancy status as an outcome.

Research on frequency of treatment with benzathine cephapirin and its effect on reproductive performance in dairy cows may also lead to more successful alternative treatment protocols for endometritis in cows. In that regard, studies to evaluate the effectiveness of combined PGF2 α or its analogues treatment and benzathine cephapirin on endometritis are needed. Studies of the effect of benzathine cephapirin treatment on specific types of bacterial infections with or without PGF2 α or its analogues treatment should also be initiated. However, research should focus on the improvement of our understanding of the innate immune response to bacterial contamination of the uterus as an important aspect of uterine health in postpartum cows.

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